

DEVELOPMENT AND GENETIC REGULATION OF THE NOVEL ABDOMINAL  
APPENDAGES IN THE MALE SEPSID FLY, *THEMIRA BILOBA*

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**Title**  
DEVELOPMENT AND GENETIC REGULATION OF  
THE NOVEL ABDOMINAL APPENDAGES IN THE MALE  
SEPSID FLY, *THEMIRA BILOBA*

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**MASTER OF SCIENCE**

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## ABSTRACT

Evolutionary novel structures do not have a known homology. They often provide a novel function to the organism. Some species of sepsid flies (family Sepsidae) have evolved novel abdominal appendages on the fourth segment in males and are thought to be used during mating, to stimulate the female abdomen. Controlled laboratory experiments were conducted to identify the necessity of these appendages in sepsid *Themira biloba*. Surgical manipulation of the bristle length of the appendages, specifically the manipulation of long bristles; prevented successful mating. *In-situ* hybridization was done to examine the expression of *abdominal-A* and *Abdominal-B* genes in sepsid *T. biloba*, and to determine whether their expression has deviated in forming the appendages. However, the expression domains of these genes are conserved in *T. biloba*, compared to *D. melanogaster*. Overall my research emphasizes the necessity of the abdominal appendages, and provides insight into the genetic basis of these novel structures.

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## **DEDICATION**

This thesis is dedicated to  
my parents, Kiri Banda and Chandra Herath  
my in-laws, Walter and Thilaka Henkanaththegedara  
my Husband, Sujana Henkanaththegedara  
and  
our children, Mihina and Sanuthi Henkanaththegedara

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## CHAPTER 1: GENERAL INTRODUCTION

### Evolutionary novelty

Evolutionary novel structures are morphological structures in a certain organism that do not have a known homology (Muller and Wagner 1991). In other words, they do not have a known relatedness to any structure in related taxon, but are a unique structural formation in an organism's body. These structures are considered *de novo* as they appear to evolve from nothing, or in other words, are not modifications of an existing structure. These structures do not share any evolutionary history to other ancestral structures, and have a non-homologous nature. In fact, these novel structures introduce newly acquired parts into a given phenotype, and sometimes perform a unique function in a particular organism (Lloyd 1971; Robertson et al. 2005). However, whether a preexisting genetic architecture is required for the appearance is still an open question (Muller and Newman 2005). Evolutionary novel morphologies are challenging to study as they are rare, and the best examples exist in non-model organisms.

Wing patterning is one such evolutionary novelty seen in the order Lepidoptera. Butterfly and moth wing patterns are made up of a specific arrangement of colored scales, which are produced by wing epithelial cells, and are unique to the order Lepidoptera. Both wing color and patterns play a significant role in both inter- and intra-specific communication, especially in predator defense, and in sexual selection (Silberglied 1984, Robertson et al. 2005). These novel arrangement of epithelial cells have allowed butterfly and moth wings to novel accomplish functions. Another well-known example from the insect world is the abdominal bioluminescent organ in fireflies in the order Lampyridae. A specialized ventral part of the abdominal segments of fireflies uses a luciferase-catalyzed oxidation reaction in order to emit energy in the form of light (McElroy 1985). This is a fascinating novel capability which adult fireflies use to recognize

mates and to attract prey (Lloyd 1971). Additionally, both the examples of butterflies and moths, and that of fireflies suggest that novel structures allow the organisms to improve their sexual attractiveness towards the opposite sex, and may play an important role in sexual selection.

Another evolutionary novel structure is the horns of Coleopteran scarab beetles (Moczek 2005). They are massive structures found on the head and thorax, as well as places where, normally, insects do not bear any outgrowth. They are as long as, or even longer than other appendages such as legs, and will sometimes make up more than 30% of an a beetle's body mass by doubling the length of the organism (Emlen 2001). These structures define a change in the phenotype in scarab beetles that also allow a novel function. Scarab beetles use them as weapons in male combat over females, and pupae use them to assist in eclosion from the larval cuticle during molting from larva to pupa (Moczek 2000).

Studies on novel morphologies have become more popular in recent years (Moczek 2008, Puniamoorthy et al. 2008). There are examples of novel structures that have been shown up in several organisms and they often provide new ecological opportunities or functions (Lloyd 1971, Silberglied 1984, McElroy 1985, Robertson et al 2005). Understanding how these novel structures evolve in organisms is important in understanding the diversity among closely related organisms, and also is important in contemporary evolutionary biology. In addition, the origin of novel structures is integrated with a wide range of biological tiers from the molecular level to an ecological change through developmental, morphological, functional, and behavioral changes (Shubin 2002, Hall and Kerney 2012). It has been suggested that the basis of evolutionary novelty is one of the main question between evolution and development of organisms due to the importance (Wagner and Lynch 2010). Therefore understanding the origin of new body plans, understanding the mechanism responsible for the new morphologies, and discovering the driving

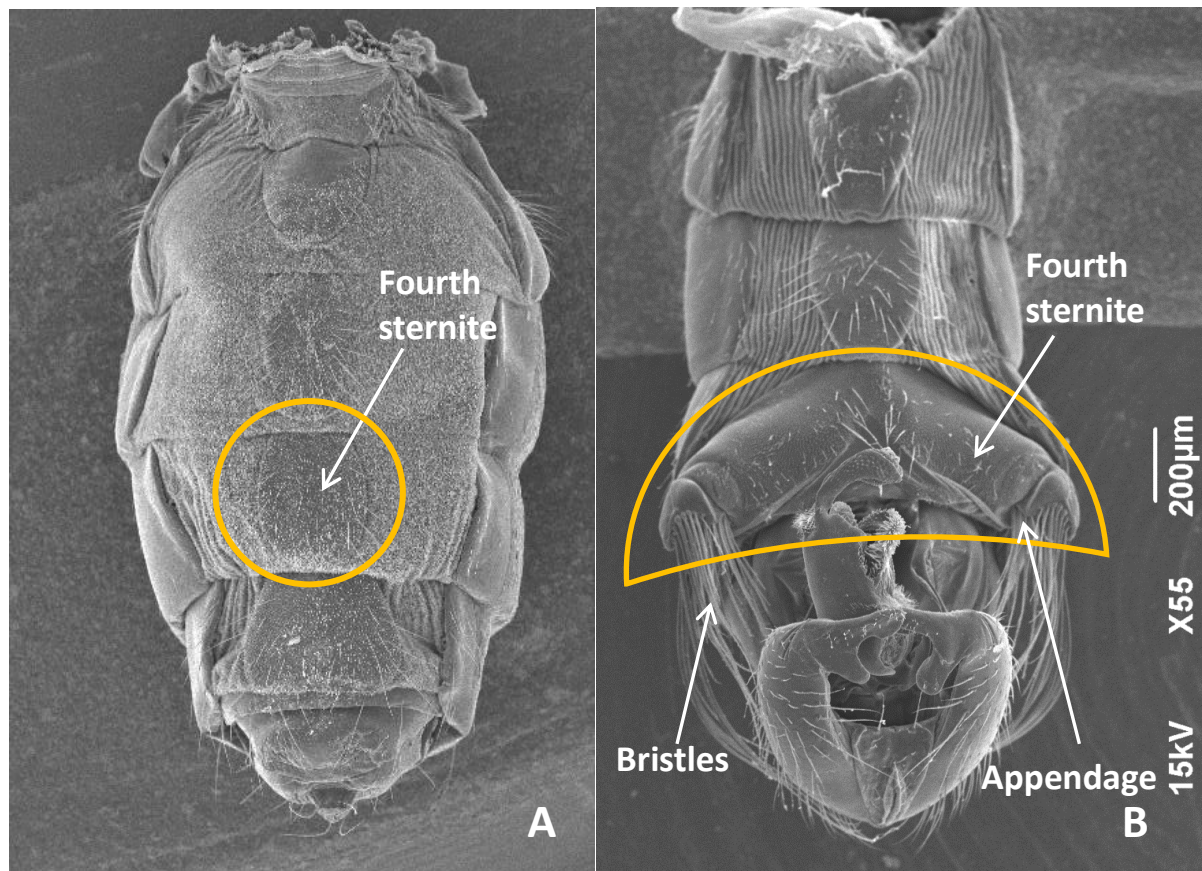
force of these morphologies are important. These findings will help to solve the questions regarding novelty in the context of evolution and development.

### **Why study Sepsid flies?**

During evolution, abdominal appendages on the first through seventh segments were lost in the basal insect body plan (Snodgrass 1935). Larvae of holometabolous insects, such as the caterpillars of butterflies, have abdominal appendages, which are called ‘prolegs’. However, a distinct characteristic of holometabolous insects is the absence of abdominal appendages in the adult stage, except genitalia and cerci. Genitalia are at the eighth and/or ninth segment and the cerci are at the tenth or eleventh abdominal segment (Matsuda 1976). Additionally, these appendages are serially homologous to other insect appendages. In other words, these abdominal appendages share the same evolutionary history with other insect appendages such as legs and antennae of the same organism (Muller and Wagner 1991, 2003). In contrast, some of the male Sepsid flies (order Diptera; family Sepsidae) possess novel abdominal appendages on their fourth abdominal segment, where normally insects do not have appendages (Pont 1979, Eberhard 2001; Figure 1.1). These abdominal appendages are very diverse in form, paired, rotational, and jointed. Further, these appendages are thought to be used by males before and during copulation to stimulate the female abdomen (Eberhard 2001; Figure 1.2).

Sepsidae is a small fly family with approximately 320 species in 32 genera (Ozerov A. L. 2005). Sepsid flies are also called black scavenger flies. Sepsid flies are globally distributed, and can be found at decaying organic matter (Pont and Meier 2002). Sepsid flies use decaying organic matter as their breeding substrate, and sepsid larvae use organic matter as a feeding source. They also have a shorter life cycle, and are easy to handle and breed in normal laboratory conditions (Pont and Meier 2002). Furthermore, sepsid flies are closely related to *Drosophila*

(Wiegmann et al. 2003) with a similar development and life cycle to *Drosophila*. Therefore, basic laboratory techniques of *Drosophila* can be easily adapted for sepsids.

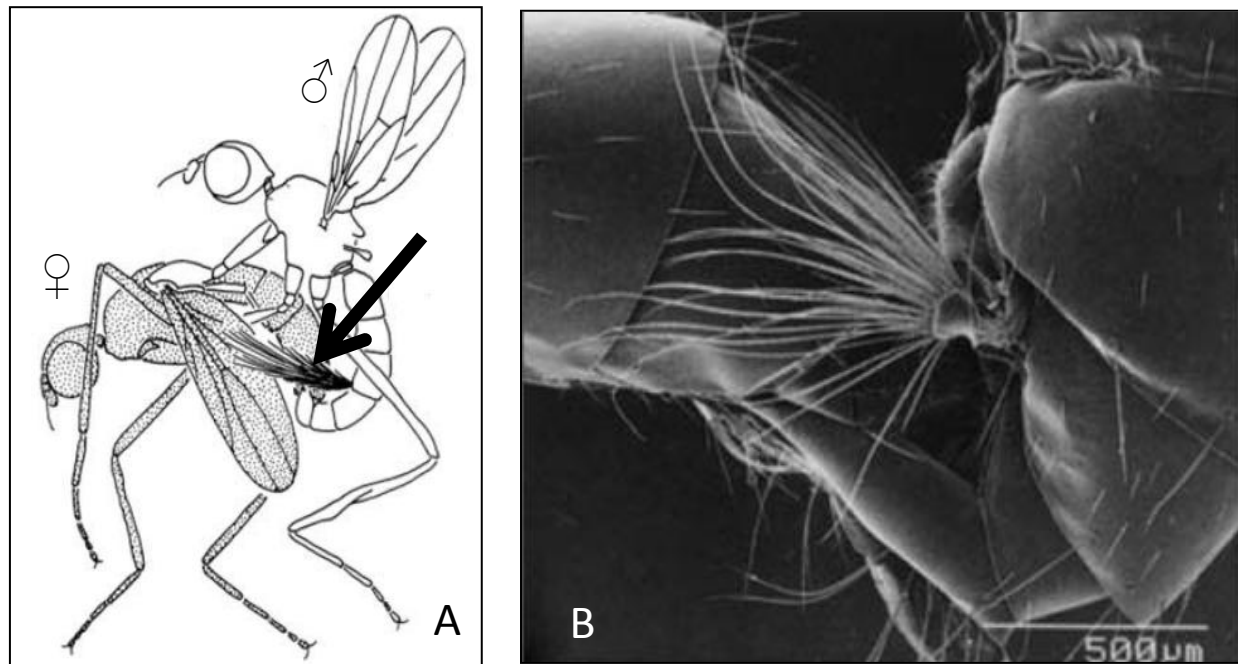


**Figure 1.1:** Abdominal appendages of the sepsid *Themira biloba* scanning electron microscopic view of (A) ventral abdomen of a female. The Figure shows that in females, all sternites are similar in size and shape. (B) Ventral abdomen of the male. Fourth sternite is marked in yellow, and male abdominal appendages with bristles are at the distal ends of the sternite (SEM-© Julia Bowsher).

Sepsid flies provide a good model organism to study evolutionary novelty for several reasons. Abdominal appendages in sepsid flies have been evolved multiple times in the Sepsidae family primarily in closely related lineages (Eberhard 2001, Wagner and Muller 2002, Bowsher et al. 2012). They have an evolutionary history of gain, loss, and a secondary gain of

these appendages, allowing a genetic comparison of the evolution of sepsid abdominal appendages (Bowsher et al. 2012). In addition, they have evolved very recently. Therefore, comparison studies between basal species without abdominal appendages and species with abdominal appendages are possible. Sepsid appendages are a fine example to study the evolution of novel structures, and also provide insights to evolutionary developmental biology. Due to their position in the phylogeny within Diptera (Wiegmann et al. 2011), understanding the evolution of novelty in sepsids will open doors to understanding the comparative development of the order Diptera. As these appendages have evolved multiple times throughout the family, understanding the developmental basics of each instance might help to understand the mechanism behind the evolution of the trait.

Even though these abdominal appendages show a morphological similarity to other insect appendages, they do not share the same evolutionary history with them. In order to form this novel structure, extensive musculature is present both inside the appendage and extending from the appendage to the ventral midline of the abdomen (Bowsher and Nijhout 2007). There are also some morphological changes, especially nearby area of the appendages such as changes in the appearance of the sternite that bears the appendages. These appendages are jointed and bear large bristles on the distal ends. Female sepsids, who do not possess abdominal appendages, do not show a morphological difference in the fourth sternite compared to the other sternites (Figure 1A). There is a significant diversity in these appendages among sepsid species, with abdominal appendages, but it is no more than a slight difference in shape, size, or bristle number in male appendages (Eberhard 2001, Pont and Meier 2002, Bowsher et al. 2012; Figure 1.2).



**Figure 1.2:** (A) Schematic view of the Male sepsid flies (*Themira* sp.) using a pair of abdominal appendages to stimulate females during copulation. The arrow indicates the bristles, which are at the end of appendages, stimulating the female (adapted from Eberhard, 2001). (B) A copulating pair where the male's fourth abdominal sternite presses forcefully against the female's tergite (Adapted from Eberhard 2001).

Bowsher et al. (2012) compared the morphologies of three different sepsid species that have abdominal appendages (*Themira biloba*, *Perochaeta dikowi*, and *Meroplus fasciculatus*) as well as a species that does not have abdominal appendages (*Sepsis punctum*). *T. biloba*, a species with abdominal appendages, possesses an enlarged, elongated fourth sternite shaped as a boomerang, and appendages with a combination of long and short bristles. *M. fasciculatus* have a relatively small sternite with larger appendages with long bristles. In addition, phylogenetic analysis shows that *T. biloba* and *M. fasciculatus* abdominal appendages are homologous to each other. Even though the abdominal appendages of males of *P. dikowi* are similar to other two species above, and they are located on either the fourth or fifth abdominal segment, which is slightly different than the arrangement in above two species (Bowsher et al. 2012).

Different aspects of sepsid flies have been studied in the past. Sepsid flies show an extremely diverse mating behavior, and most of the species have their own mating behavior element when compared to others. Sepsid mating behavior is also highly selected as same as their morphological characteristics (Puniamoorthy et al. 2009, Tan et al. 2011). Mating behavior in sepsid flies evolves faster than other morphological traits which are related to sexual dimorphism (Puniamoorthy et al. 2009).

Further, due to their sexually dimorphic characteristics, sepsid flies are an emerging model system for sexual selection. The highly modified spiny architecture of sepsid male forelegs, which help males to clamp on to females at mounting are considered a sexually selected trait (Eberhard 2001 and Puniamoorthy et al. 2008). Another interesting characteristic in female sepsid flies is when they are clamped by males; females try to avoid mating by shaking their abdomen. This female shaking may be related to mate choice (Blanckenhorn et al. 2000, Eberhard 2005, 2010). In addition to the sexual dimorphism shown by males, Puniamoorthy et al. (2010) found that the female internal genitalia are highly diverse among sepsid species. They also evolve at a much faster rate than expected (Puniamoorthy et al. 2010). Sexual dimorphism, being highly diverse in sepsid flies, it is definitely important in identifying the developmental biology of these morphological structures.

### **Sepsid abdominal appendage developmental biology**

In general, appendages in adult insects are derived from an imaginal disc (Snodgrass 1935). However, Bowsher and Nijhout (2007) showed that the abdominal appendages of the sepsid *Themira biloba* are derived from histoblast nests. There are three histoblast nests in the abdominal segments of *T. biloba*, which are referred to as the anterior dorsal, posterior dorsal, and ventral regions. According to Bowsher and Nijhout (2007), the abdominal appendages of *T.*



*biloba* are derived specifically from the ventral histoblast nest on the fourth segment, and this provides evidence for the non-homologous nature of the sepsid abdominal appendages compared to other adult insect structures. Further, cauterization of the ventral histoblast nest on one side of *T. biloba* larvae showed that the cauterized males developed ipsilateral appendages when they emerged into adults. Cauterization of the fourth ventral histoblast nest also disrupted the development of the sternite on that side and part of the pleuron. Cauterization of the genital disk resulted adults with no genitalia, but the abdominal appendages were not affected (Bowsher and Nijhout 2009). Previous research also showed that there is an increase in the histoblast cell counts in the fourth and fifth abdominal segments of male sepsid *T. biloba* by the end of last larval stage (Bowsher and Nijhout 2007).

In order to identify whether or not there is a shared developmental basis in abdominal appendage development across sepsids, Bowsher et al. (2012) compared the histoblast cell counts in the third instar larva of four sepsid species. Three of these species possess abdominal appendages, and one does not. Due to the role of histoblast cells on forming abdominal appendages, it was expected that there would be an increase in the number of histoblast cells in the fourth abdominal segment in sepsid species with abdominal appendages. The sepsid species without abdominal appendages did not show a significant difference among male and female histoblast cell counts. Indeed, all three species with abdominal appendages had a significantly higher number of ventral histoblast cells in the fourth and fifth segments compared to the other segments in male flies. In contrast, the histoblast cell counts were similar throughout all abdominal segments in female flies of two of those species. However, the third instar females of one species showed an elevation in the histoblast cell counts in the fourth abdominal segment.

## Genetic regulation of insect abdominal development

Hox genes are a set of developmental regulatory genes found in eukaryotes (Cook et al. 2001). Hox genes carry out distinct roles in the development of an organism by encoding transcription factors that control cell specification and differentiation. Hox genes are important in segment specification in arthropods and define the anterior-posterior axis (McGinnis and Krumlauf, 1992). Due to the role of Hox genes in segment specification, they are also involved in the body patterning of organisms. Moreover, evolutionary changes in the expression of Hox genes expression may have caused changes in body patterning of organisms (Akam 1995). For an example, most arthropods use thoracic appendages as a locomotive structure, but in crustaceans they are evolved as feeding structures due to a change in the *abdominal A* and *Ultrabithorax* genes (Averof and Akam 1995).

Each Hox gene is involved in regulating the expression of a variety of target genes (Botas and Auwers 1996). Therefore, mis-expression of Hox genes may alter the morphology of the organism. This suggests that changes in Hox gene expression might have influenced the evolution of body plans (Gellon and McGinnis 1998). As an example, if a gain-of-function mutation happens in the gene *Antennapedia* of the fruit fly *Drosophila*, legs will grow out of the head instead of antennae (Denell et al. 1981). It has been hypothesized that the evolution of Hox genes was the basis for the Cambrian explosion, which was marked by an endless variety of arthropods with novel phenotypic features (Valentine et al. 1999).

The bithorax complex consists of the Hox genes *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*), which are expressed from anterior to posterior (Hughes and Kaufman 2002; Figure 1.3). The expression domains of these genes broadly overlap in insects and most Hox genes interact with other Hox genes to maintain certain expression limitations. As

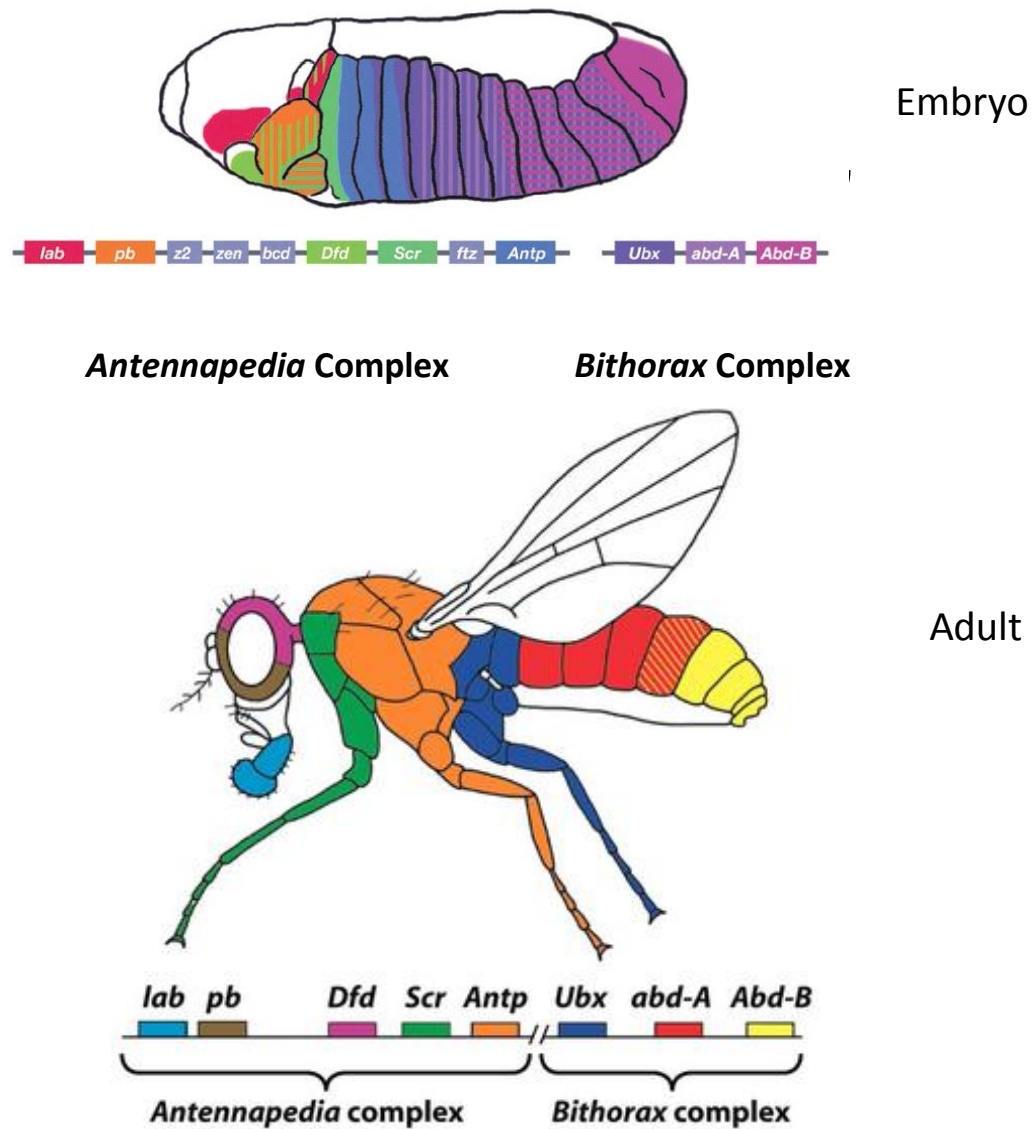
an example, mutations in *abd-A*, *Abd-B* and *esc* (extra sex combs gene) change the expression levels of *Ubx* (Struhl and White 1985).

Studies on genetic regulation of holometabolous insects growth have found that the bithorax complex gene *abd-A* is responsible for abdominal appendage suppression in holometabolous insects (Palopoli and Patel 1998; Lewis et al. 2000). Moreover, according to Vachon et al. (1992), *Ultrabithorax (Ubx)* and *abd-A*, both repress *Distal less (Dll)* in the abdominal segments in *Drosophila melanogaster*. The repression of *abd-A* by *Abd-B* in both grasshoppers and firebrats is another example of hox genes interacting with each other (Hughes and Kaufman 2002).

There is evidence that Hox genes play a role in specifying histoblasts during abdominal development in *Drosophila*, even though the exact mechanism is unclear. Simcox (1991), Casares (1996) and Estrada and Herrero (2001) demonstrated that removal of bithorax genes caused histoblasts to be absent, transformed male and female genitalia into legs or antenna, and imaginal discs formed in the abdomen. Furthermore, Jeong et al. (2006) showed that bithorax genes have been involved with the development of sexually dimorphic morphology in *Drosophila*. This previous research suggests that one or more bithorax genes may be involved in the process of differentiating the fourth abdominal segment in sepsids. Specifically, the bithorax complex of Hox genes may be responsible for defining the fourth segment in *T. biloba* through the patterning of histoblast cells, and, therefore, specify the abdominal appendages.

As no structure can be formed *de novo*, it has been suggested that novel structures can be formed from pre-existing tissues and genes or, in other words, can be co-opted from the genes that are already there (True and Carroll 2002). Co-option of existing genes and genetic pathways is a well-accepted process in formation of morphological novelty. As an example, the genes that

are involved in patterning serially homologous insect appendages such as Extradenticle (*Exd*), Distal-less (*Dll*), engrailed (*en*), and Notch have been co-opted to pattern novel abdominal appendages in sepsid *T. biloba* (Bowsher and Nijhout 2010).



**Figure 1.3:** Hox genes in *Drosophila melanogaster* embryo and adult. Shades of purple in the embryo illustrate the Bithorax complex genes in the embryo. (Adapted from Hughes and Kaufman 2002).

Notch expression specifies joint formation and *Exd* and *Dll* expression represents the presence of proximo–distal axis. Gene *en* is involved in defining anterior–posterior partition of the abdominal appendages). Bowsher and Nijhout (2010) found that the expression patterns of *Exd*, *Eng*, and Notch are at least partially co-opted to pattern the abdominal appendages. However, *Dll* was only expressed in the bristles of the developing appendages and not the proximal–distal axis of the appendage itself. This expression difference of *Dll* gene specifies a difference between sepsid appendages and normal insect appendages. Even though the basic gene level is co-opted, the appendages have been formed independently and do not bear any homology. In addition to this the bithorax complex genes *Ultrabithorax* and *abdominal-A* were found to be expressed throughout the abdominal appendage and in the surrounding epidermis. However, it is not clear whether the genes have shifted the expression domain as the used antibody recognized both genes (Bowsher and Nijhout 2010).

### **Organization of thesis**

Some sepsid species possess novel abdominal appendages in the fourth abdominal segment even though the basic insect body plan does not bear abdominal appendages up to the seventh abdominal segment. Different aspects of these novel appendages have been studied already. One aspect that has been studied was the function of the appendages. However, the research done on the necessity of these structures is minimal. Therefore, in my first part of the research, I intended to identify the necessity of these abdominal appendages for successful reproduction in sepsid flies using the sepsid *T. biloba*. I hypothesized that *T. biloba* is unable to mate without the abdominal appendages. In Chapter Two, I present manipulative experiments that were conducted to investigate the necessity of male abdominal appendages for successful breeding in sepsid *T. biloba*.

After investigating the necessity of novel abdominal appendages for sepsid *T. biloba*, I intended to identify the genetic basis of these appendages. Based on previous literature, I predicted that the three bithorax complex genes (*Ultrabithorax*, *abdominal-A*, and *Abdominal-B*) regulate the specification of abdominal appendage development in the fourth abdominal segment in *T. biloba*, and there can be a shift in the expression pattern of these three bithorax genes in order to specify abdominal appendage development. Chapter Three addresses whether the bithorax genes are involved in abdominal appendage formation, and whether the expression of the bithorax genes is shifted in order to form the appendages.

I believe my new findings will help to fill the gaps in knowledge pertaining to the areas such as identify the importance and necessity of the abdominal appendages, and identify the genetic regulation involved in specifying the abdominal appendages. The information discovered in this research may help future research on different aspects of sepsid *T. biloba* such as courtship behavior, sexual conflict, developmental evolutionary biology, and comparative biology, and possibly *T. biloba* may be used as a model system for studies on other related species.

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**CHAPTER 2: THE ROLE OF A SEXUAL ORNAMENT: THE NECESSITY  
OF A NOVEL ABDOMINAL APPENDAGE IN MATING OF THE SEPSID FLY,  
*THEMIRA BILOBA***

**Abstract**

Unlike other insects, sepsid flies have novel abdominal appendages with bristles on the fourth abdominal segment. They are thought to be used during copulation. However, the necessity of these appendages has not been directly tested. We designed and conducted controlled experiments to evaluate the necessity of the abdominal appendages in the sepsid *Themira biloba*. In the first experiment, we surgically trimmed all the bristles of the abdominal appendages of male flies and paired them with females. The number of eggs laid and the number of larvae hatched from these eggs were counted and compared with a control group. The number of eggs laid between the two groups was not significantly different, however none of the eggs hatched from the ‘bristles trimmed’ group compared to the control group. The second experiment was done where only long bristles were trimmed in a set of males, only short bristles in another set of males, and a set of control male flies were paired with females. Additionally a set of virgin females were kept by themselves as a control group. The average number of eggs laid by female flies of the “virgin” group was significantly low compared to the average number of eggs laid by the females of the other four groups. There was a significant difference of the mean number of eggs hatched among the four groups. However, eggs hatched only from the “short” and the “control” groups. According to the results, it is clear that removal of bristles, specifically removal of long bristles, of the abdominal appendage prevented successful mating in *Themira biloba*, reducing individual fitness.

## **Introduction**

Evolutionary novel structures are newly developed morphological structures which do not have a known homology (Muller and Wagner 1991). Often novel structures perform a unique function in the particular organism (Robertson et al. 2005, Lloyd 1971). Overall, novel structures signal the presence of an evolutionary change which is different from normal variation (Bowsher and Nijhout 2007, Moczek 2008).

Previous research has investigated evolutionary novelties in many organisms and has attempted to understand their ecological significance (Robertson et al. 2005, Lloyd 1971). For example, butterfly and moth wing patterning plays an important role in mate recognition, and in predator defense (Robertson et al. 2005, Silberglied 1984). Further, fireflies use their abdominal bioluminescent organs to recognize mates and to attract prey (Lloyd 1971). In both examples, the novel structure plays a major role in mate recognition. Another evolutionary novel structure is the horns of scarab beetles (Emlen 2005, Moczek 2000). These beetle horns are either enlarged mandibles or projections of cuticle in head or thorax (Eberhard 1981). Beetle horns look similar to other appendages such as antennae, mouthparts, and legs, but are much larger in size and lack muscles, nerves or joints. Scarab beetles use them as weapons in male combat over females, and pupae use them to assist in eclosion from the larval cuticle during molting from larvae to pupae (Moczek 2000). When considering the advantage of beetle horns towards adults, this novel structure provides a unique function in sexual selection among individuals. They are used as weapons during male battles to compete for females and large weapon sizes have been favored by sexual selection (Parker 1979, West Eberhard 1984).

Among these diverse occurrences of evolutionary novelties that are related to sexual selection, sepsid flies (order Diptera; family sepsidae) provide a unique opportunity to study evolutionary novel structures. In the basal insect body plan, segments one through seven do not

have abdominal appendages (Snodgrass 1935). However, male flies of some species of sepsids have abdominal appendages on their fourth abdominal segment (Pont 1979, Eberhard 2001). These abdominal appendages evolved multiple times in the family, primarily in closely related lineages (Eberhard 2001). More specifically Bowsher et al (2012) identified that the sepsid abdominal appendages evolved once, lost three times, and secondarily gained by one species. Even though these abdominal appendages show a morphological similarity to other insect appendages, sepsid abdominal appendages do not share the same evolutionary history to those appendages. Even though all other insect appendages are derived from an imaginal disc (Snodgrass 1935), sepsid abdominal appendages are derived from histoblast cells (Bowsher and Nijhout 2007). This further reinforces the non-homologous nature of sepsid abdominal appendages.

Sepsid abdominal appendages are morphologically diverse, paired, movable, rotational, and jointed, and are suspected to stimulate the female abdomen or genitalia prior to and during copulation (Eberhard 2001, Puniamoorthy et al. 2009, Tan et al. 2011). Female sepsid flies do not show a difference in the fourth sternite compared to the other sternites. However, the fourth sternite of male flies shows a morphological difference compared to the other sternites, and has jointed appendages with large bristles on the distal ends (Figure 2.1A-B). In order to form this novel structure, extensive musculature is present both inside the appendage and extending from appendage to the ventral midline of the abdomen (Eberhard 2001). As in many other organisms, sepsid species also exhibit a significant diversity in these appendages compared to other family members. This diversity includes differences in sternite shape and size, and bristle size and number (Eberhard 2001, Pont and Meier 2002, Bowsher et al. 2012).

Several studies have investigated mating behavior and how it relates to the specialized morphology of sepsid flies including courtship behavior (Ingram et al 2008, Puniamoorthy et al. 2005, 2008, and Tan et al. 2011), sexual selection on body size (Blanckenhorn et al. 2004), and morphological diversity of male ornaments (Eberhard 2003, Puniamoorthy et al. 2008, Bowsher et al 2012). Both Puniamoorthy et al. 2009 and Tan et al. 2011 have observed and videotaped the mating behavior of 28 sepsid species. Their work demonstrated that the sepsid flies show a significant morphological diversity, and fast evolving courtship diversity. Different sepsid species have a unique way of approaching opposite sexes. In addition to a diverse courtship behavior, male sepsids have heavily modified spiny architecture of their forelegs which helps them to clamp on females during copulation at mounting (Pont and Meier 2002, Eberhard 2001, Puniamoorthy et al. 2008). Another interesting characteristic was discovered by Blanckenhorn et al. (2000); the female dung fly shakes the abdomen while mating in order to avoid copulation. They have argued that this might be another mate choice characteristic. On the other hand, some sepsid species show a post copulatory mate guarding by males and less female resistance to mating (Martin and Hosken 2004). Sepsid flies are an emerging model system for sexual selection studies due to their sexual dimorphic characteristics and complex mating behaviors.

Forelegs and genitalia of male sepsid flies have assigned a strong sexual dimorphism in sepsid flies (Pont and Meier 2002). According to Eberhard (2001) a pre-mating courtship ritual has selected this sexual dimorphic fourth abdominal sternite with abdominal appendages. Therefore, it assumes that the male novel abdominal appendages in sepsid flies play a vital role in their reproduction and provide important fitness benefits. The necessity of these appendages to *T. biloba* reproduction, specifically for mating, has not been tested directly. We hypothesized that these novel abdominal appendages are necessary for the mating of sepsid species that have

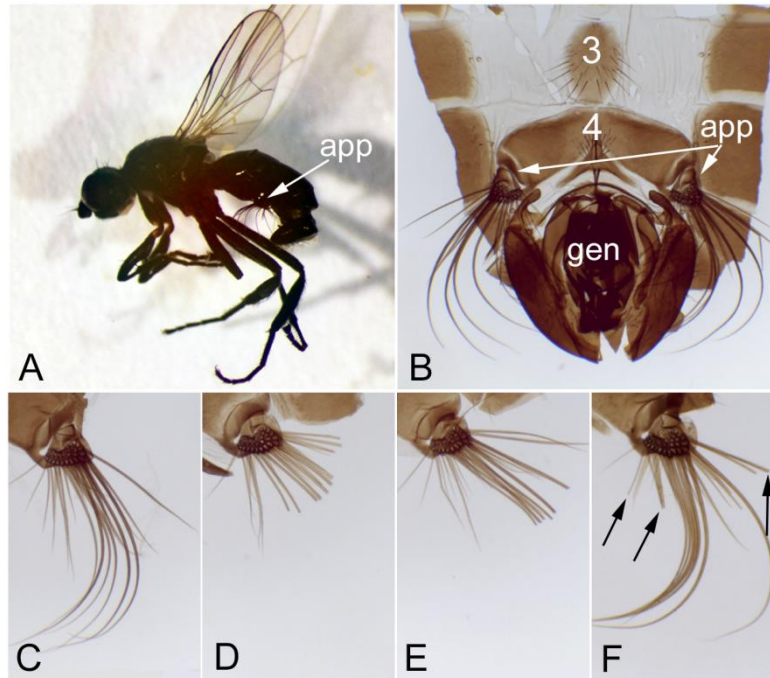
abdominal appendages, using *T. biloba* as a model. We tested the necessity of the male *T. biloba* abdominal appendages for successful mating by manipulating bristle number and length, and measuring reproductive success.

## **Methods**

### **Maintenance of fly cultures in the lab**

*Themira biloba* adult individuals were imported from the Rudolf Meier stocks at the National University of Singapore (APHIS permit # 48347). Fly raising methods were adapted from Lachmann (1991). Fly cultures were kept in an incubator at 25°C with a 16:8 hour light: dark cycle. The adult flies were given a piece of cotton soaked with sucrose solution as a food source (honey: water; 1:4), and a cow dung and agar as a food source and breeding substrate. Cow dung and agar were supplied in petri dishes. Petri dishes were filled with a 0.5 cm of agar mixed with soy-based infant formula (ProSobee LIPIL, Enfamil, Mead Johnson Co., Evansville, IN), and a 1.0 cm of cow dung layer on the top of agar. Cow dung was previously frozen at -80°C to kill any unwanted insects. Sugar solution (1:4) was supplied using a piece of cotton. Adult flies were maintained in 1 gallon plastic jars containing food source and breeding substrate. Jars were laid on their side, and a damp cotton layer was placed at the bottom of the jars to keep the jars moistened. The Petri dishes with eggs were collected on a daily basis. Larvae were raised in these petri dishes, which were placed inside lidded disposable food containers (Ziploc brand) punched with air holes. Cow dung and agar were provided as needed. Feeding was terminated when the first batch of larvae started to pupate.





**Figure 2.1:** Microscopic views of the abdominal appendages with bristles in *T. biloba* and the bristles after surgical manipulations. (A) The appearance of abdominal appendages with bristles in *T. biloba*. The white arrow shows the location of the appendage with bristles. (B) An enlarged view of the abdominal appendages with bristles at each side of the 4<sup>th</sup> sternite. (C) Bristles of the Control group where none of the bristles are trimmed. (D) Appearance of the bristles when all the bristles are trimmed more than 50 % of the length of the longer bristles in Experiment 1 (E) Appearance of bristles when only long bristles were trimmed in Experiment 2. (F) Appearance of bristles when only short bristles were trimmed in Experiment 2. Arrows are pointing to the trimmed shorter bristles.

### **Experiment 1: The effects of removal of abdominal appendage bristles of *Themira biloba* on reproduction**

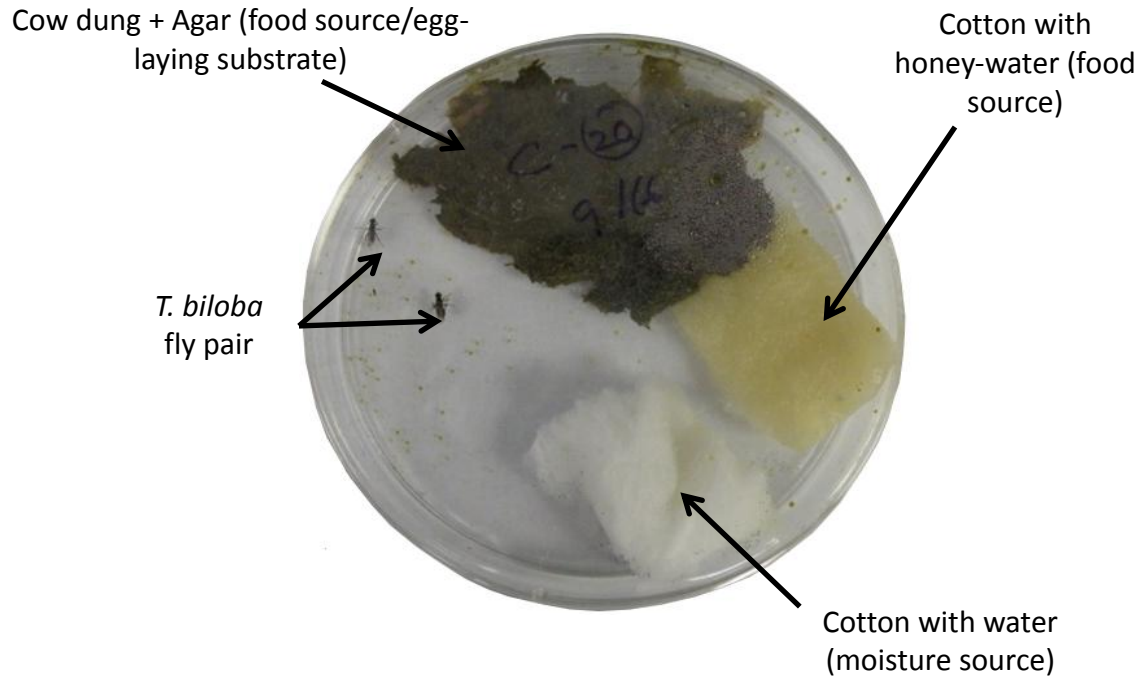
Adult flies were collected from the same generation and anesthetized using CO<sub>2</sub>. Sixty males and females were separated as soon as they emerged from pupae. Sexed flies were kept in disposable food containers with a piece of cotton soaked with sugar solution and a piece of well moistened cotton. The plastic containers were kept in an incubator at 25°C with a 16:8 hour light: dark cycle until the experiment.

Thirty males were randomly collected, anesthetized with CO<sub>2</sub> and the distal ends of the bristles in both left and right were trimmed using micro-scissors (Fine Science Tools), ensuring that more than 50% of the total length (of longest bristles) was cut off (Figure 2.1D). All flies with surgically removed bristles were monitored for 24 hours for any handling induced mortality before using in the experiment. The other 30 male flies were also anesthetized with CO<sub>2</sub> and handled in a similar way without removing bristles to be consistent between the flies of control and treatment groups. All treated flies (“bristles trimmed” and “control”) were maintained in larger petri dishes with a piece of cotton soaked with sugar solution and a piece of well moistened cotton. All the flies were at least two days old by the time of the experiment.

Large petri dishes (10 cm diameter) were used as experimental units. One half of each petri dish was filled with cow dung. A piece of cotton soaked with sugar solution and cotton square soaked with water to moisten inside the dish were supplied in each petri dish (Figure 2.2). A total of sixty such petri dishes were established and randomly assigned to either “bristles trimmed” treatment or control, ensuring 30 replicates for each group. Flies were anesthetized with CO<sub>2</sub> to minimize handling stress and introduced in to petri dishes as soon as possible. Minimal time was used to introduce the flies into petri dishes. “Bristles removed” petri dishes were assigned with a single virgin female fly and a “bristles trimmed” male fly. A single virgin female and a single male with no trimmed bristles were assigned for each “control” petri dish. Flies were monitored for approximately 10 minutes and any dead flies were replaced with another appropriate fly (same sex, same age, and same treatment).

The petri dishes with flies were placed in an incubator at 25°C and a 16:8 h light–dark cycle which is the same condition as the lab population. Petri dishes were examined for eggs after 48-72 hours and adult flies were removed upon the presence of eggs. Eggs were counted

under 3X magnification of a dissecting microscope (Leica) and recorded. Subsequently, these eggs were incubated for another 48 hours and scored for hatching. Embryonic development in this species takes approximately 24 hours at 25°C, thus 48 hrs is sufficient time to allow all fertilized eggs to hatch.



**Figure 2.2:** The setup of the experimental unit. Each fly pair was provided with cow dung, honey water and a moisture source. Each experimental unit was placed in a 25°C incubator.

Data were analyzed using R statistical software program (R Development Core Team, 2010). Shapiro-Wilk normality test (function *shapiro.test*) was used to test the normality of data. Since the data were skewed (total number of eggs:  $W=0.9$ ,  $p\text{-value}<0.0001$ ; total number of eggs hatched:  $W=0.7157$ ,  $p\text{-value}=1.804e-09$ ), the Wilcoxon rank sum test (function *wilcox.test*) was performed on the data of the “bristles removed” and “bristles not removed” groups.

## **Experiment 2: The effects of reduction of the length of short bristles versus long bristles of *Themira biloba* on mating**

The bristles in *T. biloba* abdominal appendages consist of a bunch of short and long bristles. Therefore, another experiment was set up to identify which bristles are responsible for the mating of adult flies. The experimental set-up was the same as the first experiment stated above. Here we had four main groups: (1) “Control,” where females paired with normal males with bristles, (2) “Short,” where females paired with short bristles removed males, (3) “Long,” where females paired with long bristles removed males, and (4) “Virgin,” where only females are by themselves (Figure 2.1C, E, F). Each group had 20 flies of each sex except for the virgin females group. 20 virgin females were maintained in isolation throughout the experiment.

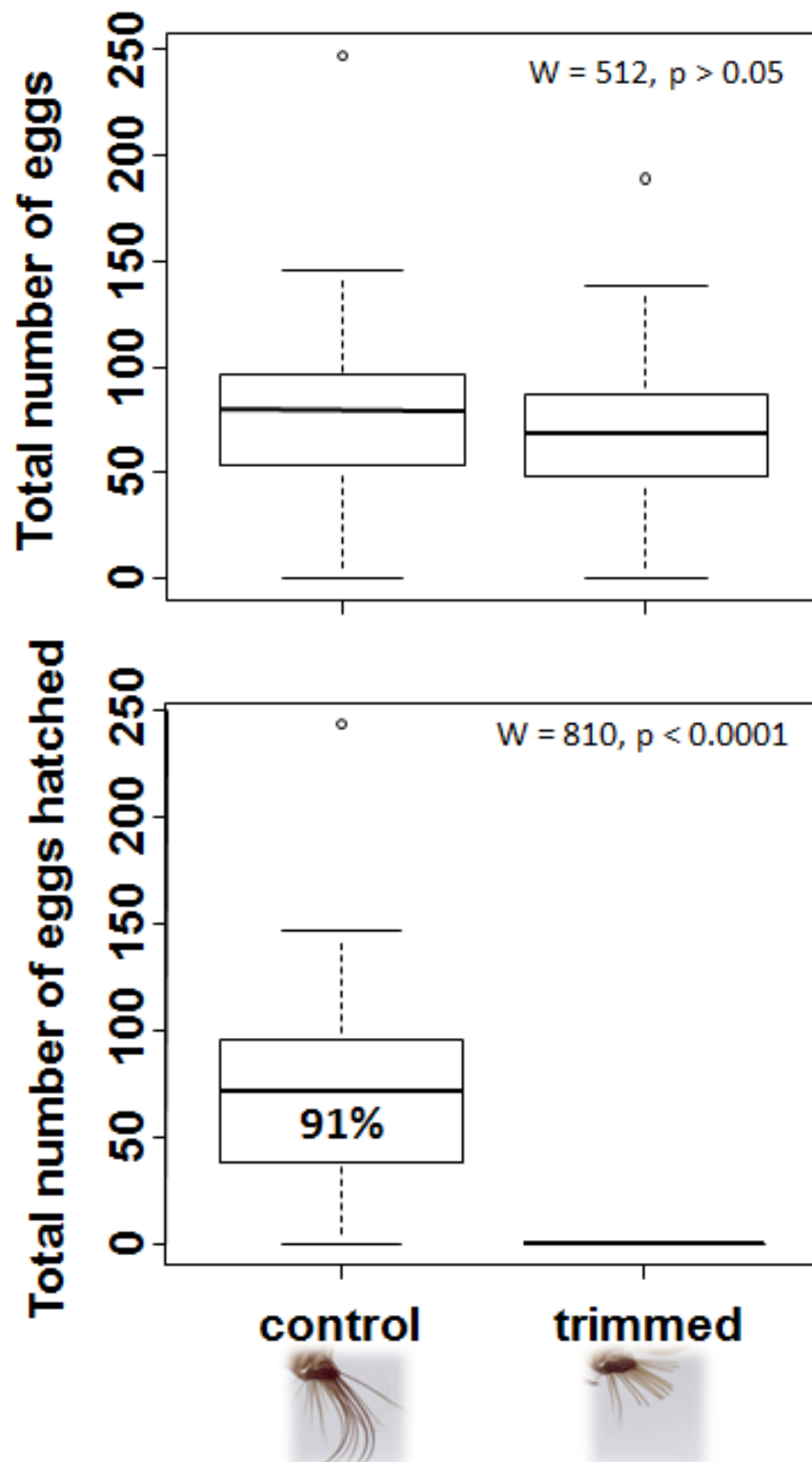
Flies were maintained and experimental units were made in the same way as in Experiment 1. All flies were monitored for approximately 10 minutes and any dead flies were replaced with another appropriate fly (same sex, same age, and same treatment). The petri dishes with flies were kept in the same environmental condition as in Experiment 1. After 48-72 hours the petri dishes were examined for eggs, and adult flies were removed if eggs were present. Eggs were counted and recorded, and they were incubated. The number of hatched eggs was recorded after 48 hours.

Shapiro-Wilk normality test (function *shapiro.test*) was used to test the normality of data. Since the data were skewed, Kruskal-Wallis H test (function *kruskal.test*) was used to compare the mean group differences of total number of eggs laid and total number of eggs hatched. A post-hoc pairwise comparison was done using Tukey HSD test (function *TukeyHSD*) after a significant Kruskal-Wallis H test (Zar 2012) to identify significantly different group mean comparisons.

## Results

### **Experiment 1: The effects of removal of abdominal appendage bristles of *Themira biloba* on mating**

The number of eggs laid in the treatment group (i.e. “bristles trimmed”) was not significantly different from the control flies ( $W = 512$ ,  $p\text{-value} = 0.3629$ ; Figure. 2.3). The average number of eggs laid by “control” group (76 eggs  $\pm 8.90$ ) and the eggs laid by “bristles trimmed” group (69  $\pm 6.68$ ). However, the average number of eggs hatched in the “bristles trimmed” group compared to the control ( $W = 810$ ,  $p\text{-value} = 1.943\text{e-}09$ ; Figure. 2.3). No eggs hatched (0 eggs  $\pm 0$ ) in the “bristles trimmed” group compared to an average of (69.03 eggs  $\pm 9.33$ ) in the control (Figure. 2.3). 91% of the eggs hatched in “control” group, and 0% of the eggs hatched in the “bristles trimmed” group out of the total number of eggs laid.



**Figure 2.3:** The total number of eggs (a) and the total number of eggs hatched (b) for female *Themira biloba* of two different groups. The two groups represent females paired up with bristles untrimmed males (control) and females paired up bristles trimmed males (trimmed).

## **Experiment 2: The effects of reduction of the length of short bristles versus long bristles of *T. biloba* on mating**

Female flies of all four groups (control, short, long, virgin) laid eggs. However, there was a significant difference of the mean number of eggs laid among the four groups ( $X^2 = 32.3517$ ;  $df = 3$ ;  $p\text{-value} = 4.412e-07$ ; Figure. 2.4). Tukey HSD post-hoc analysis showed that the female flies of the “virgin” group laid a significantly lower average number of eggs ( $16.05 \pm 4.42$ ) compared to the average number of eggs laid by the females of “short” ( $50.95 \pm 2.60$ ), “long” ( $46.48 \pm 3.84$ ) and “control” ( $55.62 \pm 3.84$ ) groups (Figure. 2.4; Table 2.1).

There was a significant difference in the mean number of eggs hatched among the four groups ( $X^2 = 71.7453$ ;  $df = 3$ ;  $p\text{-value} = 1.805e-15$ ; Figure. 2.4). The majority of the eggs from “control” and “short” groups hatched into larvae. The eggs hatched from the group “short” was  $47.67 \pm 3.59$ , and the group “control” was  $55.61 \pm 3.84$ . In contrast, the average numbers of eggs hatched from “virgin” and “long” groups were zero. Tukey HSD post-hoc analysis showed that the average number of eggs hatched from “control” and “short” were significantly higher compared to the average number of eggs hatched from “long” and “virgin” groups (Figure. 2.4; Table 2.2). 100% of the eggs hatched in the “control” group, 94% of the eggs hatched from the “short” group, and 0% of the eggs hatched in “Long” and “Virgin” groups out of the total number of eggs laid.

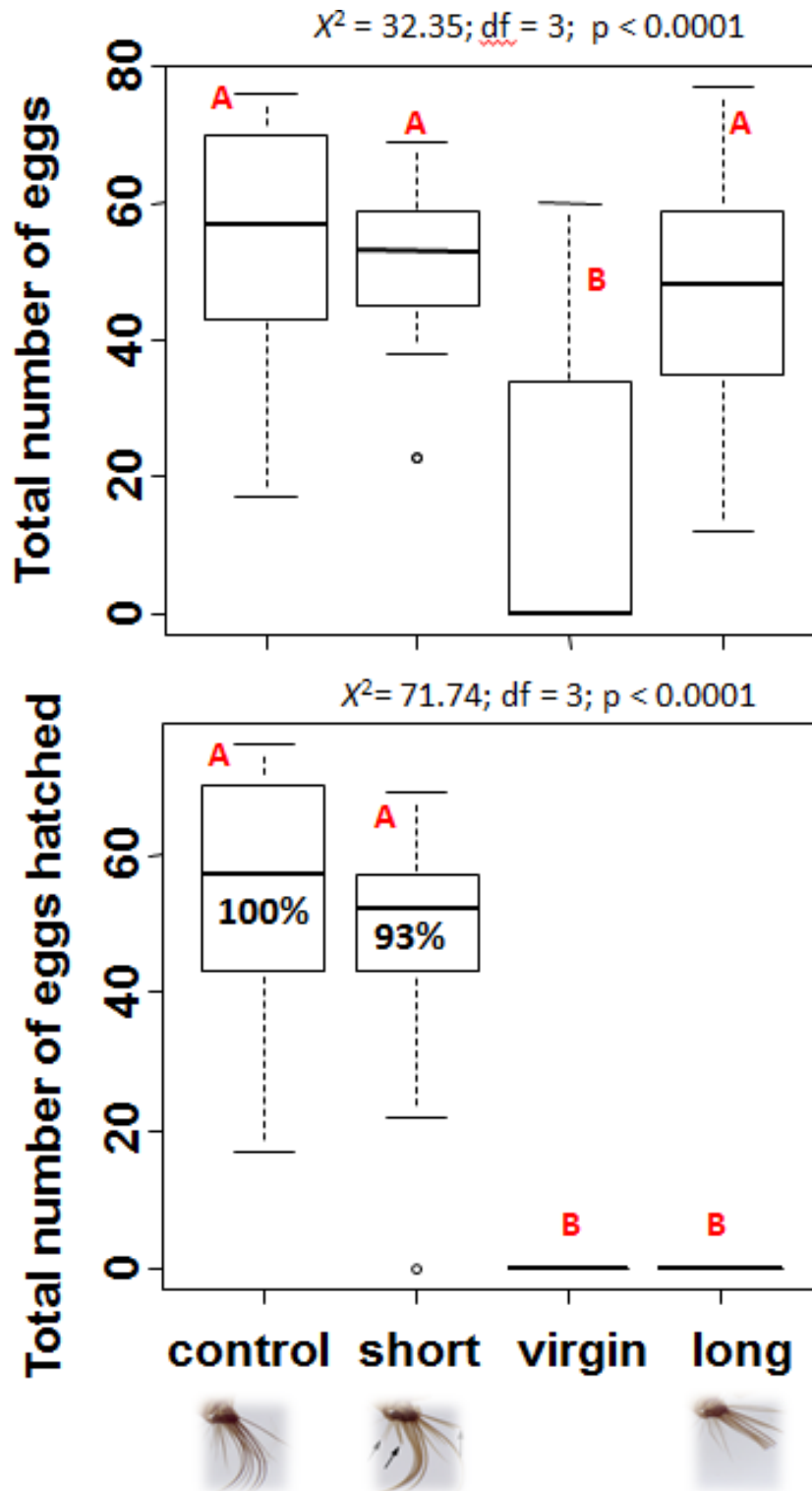
**Table 2.1:** Tukey HSD post-hoc pairwise comparisons of the average total number of eggs laid by female *Themira biloba*.

Treatment pair	Adjusted p-value
Long -Control	0.3348
Short -Control	0.8094
Virgin-Control	0.0000
Short -Long	0.8541
Virgin-Long	0.0000
Virgin-Short	0.0000

**Table 2.2:** Tukey HSD post-hoc pairwise comparisons of the average number of eggs hatched of female *Themira biloba*.

Treatment pair	Adjusted p-value
Long -Control	0.0000
Short -Control	0.4046
Virgin-Control	0.0000
Short -Long	0.0000
Virgin-Long	1.0000
Virgin-Short	0.0000





**Figure 2.4:** The total number of eggs laid (a) and the total number of eggs hatched (b) for female *Themira biloba* of four different groups. The four groups represent virgin females (virgin), females paired up with short-trimmed males (short), females paired up with long-trimmed males (long) and females paired up with untrimmed males (control). Similar upper case letters above each bar indicate non-significant post-hoc pairwise comparisons.

## Discussion

The novel abdominal appendages in sepsid flies are used before and during copulation by male flies to caress the female abdomen (Eberhard 2001, Puniamoorthy et al. 2008, 2009, Tan et al. 2011). However the necessity of these novel abdominal appendages to sepsid flies remained unidentified. Therefore, we tried to investigate the necessity of the abdominal appendages using sepsid *T. biloba*. We manipulated the bristles at the end of the abdominal appendages and tested for successful mating in *T. biloba*.

In the first experiment, when all the bristles of the abdominal appendages were removed, females were able to lay eggs and there was not a significant difference between control group and bristles trimmed group in the number of eggs laid. However, as the eggs from the “bristles trimmed” group did not hatch, that depicts that these females did not get a chance to mate with their partner. In other words, removal of bristles avoided successful reproduction in *T. biloba*. This also suggests that bristles in males have an essential function in mating, and abdominal appendages in male *T. biloba* are an essential element for copulation and successful breeding of the species.

How does bristle length affect mating? Are there any specific types of bristles or a particular bristle length that is important? These questions were answered by the second part of the study. As *T. biloba* bristles consist of short and long bristles, identifying which bristles are significant or necessary for the mating of *T. biloba* was important. In Experiment 2, trimming only the long bristles on both sides avoided successful mating in *T. biloba*. These females were capable of laying eggs which were apparently unfertilized as none hatched, but avoided mating with the long bristles trimmed male. However, when only the short bristles were trimmed in male *T. biloba*, the females could mate with their partner and produced fertilized eggs. The

number of eggs laid and number of eggs hatched between control group and short bristles trimmed group were not significantly different. These findings suggest that longer bristles are necessary for mating in *T. biloba* and have been sexually selected. In other words, females favored males with long bristles compared to the short bristle males.

The number of eggs laid by females in control and bristles trimmed groups in Experiment 1 were not significantly different. At the same time, females of control, short bristles trimmed, and long bristles trimmed groups in Experiment 2 (where a bristles trimmed or not trimmed male was present) laid a significantly similar number of eggs. These results suggest that the surgical trimming of bristles did not affect the activity level of the bristles trimmed males.

*T. biloba* individuals have various lengths of bristles. So, there is a possibility to think that larger males might have longer bristles and vice versa. However, some studies done by Josh Johnson (unpublished data) in Bowsher lab showed that the bristle length does not correlate with the size of the male.

Additionally, our experiment also showed that female *T. biloba* are capable of laying unfertilized eggs. Therefore, even though the females do not mate, they will lay eggs. However, the average number of eggs laid by a female in “virgin” group (16.05  $\pm$  4.42) is significantly lower than the number of eggs laid by a female in “control” (55.62  $\pm$  3.84), “long bristles trimmed” (46.48  $\pm$  3.84), and “short bristles trimmed” groups (50.95  $\pm$  2.60) (Figure. 2.4; Table 2.1). However, this result raises another question such as, “Will females receive chemical cues if a male is around (even though the mating does not occur)?”, further, “will these females lay more eggs compared to when a male is not around”? This will not affect the population; however it is an interesting question.

Because novel abdominal appendages are a necessity for sepsid flies, it is important to identify what is the driving force in formation of these structures. Sepsid flies are used as a model system for sexual selection studies due to their various sexual dimorphic characteristics and elaborative mating behavior (Ang et al. 2008; Ingram et al. 2008; Puniamoorthy et al. 2008, 2009, 2012). Even though the *T. biloba* abdominal appendage is a non-genital structure, according to their function, it is reasonable to consider them as a supplementary genital structure. Sometimes entomologists have included non genitalic structures which are not connected to the segment of genitalia, as ‘genitalia’ as they directly contact with females during copulation (Wood 1991). For example the antennae in crustaceans, anterior legs of spiders, and head, mandibles, antennae, cerci, and wings of insects all can be considered genital structures as they have been modified to grasp the female during copulation (Eberhard 2010). On the other hand, genital structures show a broad diversity even in closely related species, and do evolve rapidly compared to other organs (Arnquist and Rowe 2002, Hosken and Stockley 2004, Takami and Sota 2007). Recent studies show that sexual selection is an appropriate candidate for being the driving force towards genital divergence (Eberhard 1985, 2001; Hosken and Stockley 2004). Therefore, when considering the function of sepsid novel abdominal appendages, the relationship between non-genitalic structures and genitalic structures, and the driving force of genitalic structures; we can argue that, sexual selection can be a possible candidate which drives the evolution of the novel abdominal appendages in sepsid flies as well.

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## **CHAPTER 3: GENETIC REGULATION OF THE NOVEL ABDOMINAL APPENDAGES IN MALE SEPSID FLY, *THEMIRA BILOBA***

### **Abstract**

Abdominal appendages in male sepsid flies are considered an evolutionary novel structure due to their non-homologous nature. These structures are used by male flies during and after copulation. However, the genetic regulation involved in forming this complex morphology has not been identified. Hox genes play a major role in segment specification in arthropods, defining anterior-posterior axis of embryos, and regulating the expression of a variety of target genes. According to their role in segment specification, I proposed that the Bithorax complex Hox genes are potential candidates for the development of abdominal appendages in sepsid *Themira biloba*. I hypothesized that a shift in the expression domain of one of the three bithorax genes *Ultrabithorax*, *abdominal-A* and *Abdominal-B* may occur at the fourth abdominal segment making the phenotype different in the sepsid fly *T. biloba*. However, the results suggest that there has been no change in the expression pattern of the genes *abdominal-A* and *Abdominal-B* in *T. biloba*.

### **Introduction**

Novel structures are a new phenotype in a particular organism with a unique function. Male flies of some sepsid species retain abdominal appendages where normally insects do not bear appendages (Pont 1979, Eberhard 2001). These appendages are used at mating in sepsid flies. Previous research findings stated in the second chapter showed that the novel abdominal appendages in sepsid *T. biloba* are necessary for their mating process. Research on the developmental specification of these appendages is minimal. Therefore, identifying the genes that are responsible for forming these novel structures is very important, and may provide

insights in evolutionary developmental biology. The genes that make each body parts are highly conserved in insects. Therefore, discovering how these genes have been used or how their expression has been changed in forming the novel structure only in some organisms may fill gaps in evolutionary developmental biology (Kelsh et al. 1993, Averof and Patel 1997, Abzahnov and Kaufman 1999).

### **Hox genes determine segment identity**

Hox genes are a set of developmental regulatory genes that are responsible for carrying out distinct roles in the development eukaryotes (Cook et al. 2001). Hox genes have a specific domain (location) in the anterior-posterior axis, and are conserved in all bilateral animals (McGinnis and Krumlauf 1992). Each Hox gene specifies the identification of a different segment, and may also combine with other Hox genes to perform this role (Palopoli and Patel 1998). Even though the way Hox genes control the segment identity is still unknown, they regulate the expression of various target genes that determine cell fate in organisms (Botas and Auwers 1996). A change in expression of a single Hox gene may induce a change in a variety of target genes, which then change the entire morphology of the organism. Therefore, changes in Hox gene expression might have influenced the evolution of body plans (Wagner-Bronholz et al. 1991, Carroll 1995, Gellon and McGinnis 1998).

Changes in Hox genes are responsible for the morphological differences between closely related organisms. The differential development of wing and haltere in *Drosophila* is controlled by the gene *Ultrabithorax (Ubx)*. Haltere are a modification of hindwings and function as a balancing structure. *Ubx* is expressed in the development in haltere, but not expressed in the wing (Struhl 1982). Weatherbee et al. (1998) showed that *Drosophila* haltere development is repressed by *Ubx* gene. *Ubx* regulates target genes which act at different stages of wing

patterning pathway. However, in butterfly hindwings except for *Distal less (Dll)* gene other genes are not repressed by *Ubx* gene. *Dll* expression is regulated by *Ubx* in an exceptional way in butterflies (Weatherbee et al. 1999). Changes in expression of Hox genes in each organism may have made these organisms different from each other. Another example of the change in Hox expression is the treehopper ‘helmet’ which has been modified from wing appendage. This is a modification in treehoppers which was caused by not repressing the appendage formation regulatory gene *Nubbin* by the normally repressing gene *Sex combs reduced* (Prud’homme et al. 2011). At the same time, mis-expression of Hox genes causes alterations in segment identity. A gain of function mutation in the *Antennapedia* gene in *Drosophila* causes legs to grow out of the head instead of antennae (Denell et al. 1981). All these instances suggest that Hox genes are correlated with the arthropod segmental identity, morphology, and overall the altering of body forms. Therefore, I predict that Hox genes may be responsible for the specification of the novel abdominal appendages in sepsid flies.

### **Bithorax complex: possible candidates for abdominal appendage formation**

Hox genes in insects are located in two different chromosomal regions, the Antennapedia complex and the Bithorax complex. The Antennapedia complex is essential for specifying the identity of a body region from head to the anterior portion of the second thoracic segment, and the Bithorax complex (BX-C) specifies the identity of body region from the posterior portion of the second thoracic segment to the anterior portion of the ninth abdominal segment (Celniker et al. 1989). Therefore, the abdomen of insects is specified by Bithorax complex genes. Bithorax complex consists of three Hox genes: *Ultrabithorax (Ubx)*, *abdominal-A (abd-A)* and *Abdominal-B (Abd-B)* which are expressed in the given order from the anterior to the posterior of the insect abdomen. However, the expression domains of these genes broadly overlap in insects

such that multiple *Hox* genes are expressed in particular segments. Hox genes may interact with other Hox genes to maintain certain expression limitations (Vachon et al. 1992, Hughes and Kauffmann 2002; Figure 1.3). It has been identified that interactions between Bithorax genes specifically *Ubx* and *abd-A* are responsible for abdominal appendage suppression in basal insect body plan (Palopoli and Patel 1998, Lewis et al. 2000). In crustaceans, a change in *Ubx* and *abd-A* gene can be the cause of change in their anterior thoracic limbs to feeding appendages called maxillipeds (Averof and Patel 1997).

The role of Hox genes in specifying segment identity extends to the patterning of all segmental structures, not just appendages. Studies in *D. melanogaster* done by Simcox (1991), Casares (1996), and Estrada and Herrero (2001) showed that removal of bithorax genes caused histoblasts to be absent, transformed male and female genitalia into legs or antenna, and imaginal discs formed in the abdomen in *Drosophila*. Jeong et al. (2006) discovered that bithorax genes have been involved in the development of sexually dimorphic morphology in *Drosophila*. All of this information regarding Hox genes in *D. melanogaster* would suggest that one or more bithorax genes may be involved in the initial specification of the fourth abdominal segment in sepsids.

Therefore, my main interest was to Figure out whether the bithorax genes are responsible for the specification of the fourth abdominal segment as the location for the abdominal appendages in *T. biloba*. Specifically I was interested in identifying whether there is a shift in the expression domain of any of the bithorax genes, and if this could cause a change in the fourth abdominal segment characteristics in male flies in order to form the appendages. In order to see the gene expression patterns in *T. biloba* *in-situ* hybridization experiments were performed.

## Methods

### ***Themira biloba* culture handling**

*Themira biloba* adults were brought from Rudolf Meier stocks at the National University of Singapore (APHIS permit # 48347). Flies were maintained in an incubator at 25°C with a 16:8 hour light: dark cycle. Adults were fed with honey: water in 1:4 ratio. The breeding substrate was 0.5cm of agar and soy based infant formula (Prosobee LIPIL, Enfamil, Mead Johnson Co., Evansville, IN) overlaid with approximately 1.0 cm layer of cow dung. Adults were allowed to lay eggs on the dung for approximately eighteen hours, which allowed for the collection of a variety of embryonic stages.

### **Obtaining sequence for *Themira biloba* Bithorax genes**

Because no genomic or Expressed Sequence Tag (EST) resources exist for any sepsid fly, the mRNA sequence of *Ubx*, *abd-A* and *Abd-B* needed to be obtained. The sequence of *Abd-B* was already available.

In obtaining the already available sequence of *Abd-B*, the following primers were used:

F1: GCGTTTCTGCTTCGAGACAT

F2: CAGACGGAGAATCGAAATGG

R1: AAGGATCCGTCGACATCGAT

R2: CGACATCGATAAACTAGGGA

However, only partial sequences were available for *abd-A* and *Ubx*. Over 750 bp is required for successful *in situ* hybridization in *T. biloba*. Therefore, in order to obtain more sequence, a 3' Rapid Amplification of cDNA Ends Polymerase Chain Reaction (RACE PCR) was done.

A RACE PCR amplifies unknown cDNA sequences corresponding to the 3' or 5' end of RNA. RACE PCR contains reverse transcription and PCR amplification of the cDNA copies.

The primers used were as follows:

F1: CGCCCTATGCCTAACTGAGA

R1: AGGTCGTGGTTGGTCTTGTC

R2: TGCCTTCAGTAGGTCGTGGT

In order to make *T. biloba* cDNA for the RACE PCR, RNA was extracted from *T. biloba* pupae. Pupae were collected and inserted in a 1.5 ml eppendorf tube. Then 100 µl of TRIZol (Invitrogen) was added to embryos and homogenized. Then another 900 µl of TRIZol was added and incubated at room temperature for 5 minutes. Then 200µl of chloroform was added and the tube was shaken for 15 seconds and then incubated for another 2-3 minutes at room temperature. The tubes were centrifuged at 12,000 rpm for 15 minutes. Then the aqueous layer was removed and put in a new tube. 500 µl isopropyl alcohol was added to the tube and incubated at room temperature for 10 minutes. The tubes were centrifuged at 12,000 rpm for 10 minutes. The gel like pellet was left and the supernatant was removed. The pellet was washed once with 1 ml 75% EtOH in DEPC treated water. Then the tubes were vortexed and centrifuged for 5 minutes at 7000 rpm. Alcohol was removed as much as possible without letting the pellet dry out. Next 15 µl of DEPC treated water was added and vortexed to resuspend the pellet. Then the RNA was stored in the freezer overnight. In order to make cDNA the protoscript cDNA synthesis kit was used with RACE polyT primer. The RNA template and the primer were incubated for 5 minutes at 70°C. Then the master mix that consisted of 5X buffer, MgCl<sub>2</sub>, dNTPs, RNase inhibitor, RT enzyme, and PCR H<sub>2</sub>O was added. The cDNA was synthesized following manufacture instructions.

The cDNA made from *T. biloba* pupae was used to do a RACE PCR with gene specific primer combinations to obtain a longer sequence. Successfully amplified samples were selected, and were then purified (*illustra GFX PCR DNA and Gel Band Purification Kit*, GE Healthcare). The purified samples were then cloned into a vector (pGEM® –T and pGEM®-T Easy Vector Systems, Promega). A colony PCR was used to analyze the presence of the inserts from the amplified samples. Possible inserts were selected and sequenced at the University of Arizona Genetics Core. A long enough sequence with 925 bp was created by combining this sequence (508 bp) along with the already available *abd-A* sequence with 417 bp.

Many attempts were taken by several people, with different approaches such as RACE PCR, Touchdown PCR, and using a 454 sequencing transcriptome to obtain a long enough sequence for *Ubx* gene in order to create a probe, but all attempts failed to produce a sequence long enough to make a probe for *in-situ* hybridization.

### ***In-situ* hybridization**

The sequence obtained for *abd-A* was used to make a probe for *abd-A* according to DIG labeled RNA probe synthesis protocol. First, the required template was amplified from the plasmid using a Colony PCR, and then analyzed on a gel. After confirming that the gel band was the same size as the cloned fragment, the DNA was purified. Then the product was quantified using Nano Drop. Then the DIG labeling reaction was set up with template, DIG NTP, RNasin, 10X buffer, polymerase (either SP6 or T7), and H<sub>2</sub>O. All the solutions were kept RNase free. Then the reaction mixes were incubated at 37°C for 120 minutes. 1/10 volume of 4M LiCl was added to the reaction mix followed by a three times volume of ice cold EtOH. Then reaction mixes were incubated at 20°C at 120 minutes. The samples were spinned at 12000 rpm for 15 minutes at 4°C. The pellet was washed with 70% ice cold EtOH in DEPC treated water. Then the

samples were spun for 5 minutes at 4°C and briefly let sit for 5 minutes at room temperature to dry. The pellet was re-suspended in 50 µl hybridization buffer and stored at -20°C.

This *abd-A* probe and the already available *Abd-B* probe (Bowsher, unpublished) were used to assay the expression of the *abd-A* and *Abd-B* genes using in-situ hybridization. A specific mRNA sequence in tissue will get localized in *in-situ* hybridization by hybridizing the complementary strand of a nucleotide probe to the sequence of interest. Therefore, a labeled anti-sense mRNA probe sequence that is complementary to the sequence of specific mRNA will hybridize with the mRNA and help visualize the specific cells that express it.

Embryos laid over an 18 hour window were rinsed several times with egg wash solution, then placed in 50% bleach and egg wash solution for approximately 1 minute to remove the chorion. Then again embryos were rinsed with egg wash solution well to remove any bleach. The embryos were transferred into a glass scintillation vial with 1:1 solution of heptane: PEM-FA fixative (3.7% FA). The embryos were agitated vigorously for 20 minutes on a rotator. The embryos were removed from the fix- heptane interface with a pasture pipet, and placed in a new scintillation vial. An equal amount of 100% methanol was added to n-heptane and shaken vigorously to crack the vitelline membrane. Embryos were washed multiple times in methanol to remove traces of heptane. Embryos were stored in methanol at -20°C.

Prior to hybridization, embryos were pretreated to increase permeability. Embryos were rehydrated from methanol into PBST. Various washes with 7:3, 1:1, and 3:7 MeOH: PBST were done for 5 minutes, and a 100% PBST wash was done 6 times 5 minutes each time. Then the embryos were treated with non-predigested Proteinase K (1:1000 dilution with PBST). Then a 2 minute PBST wash followed by two times for five minutes washes. The embryos were re-fixed in 4% paraformaldehyde/ 1X PBS for 20 minutes while shaking. The re-fixed embryos were



washed five times for five minutes. While these washes were taking place, embryos were separated into individual 1.5 ml eppendorf tubes for hybridization with different probes. Embryos were equilibrated in 1:1 PBST: Hybridization Buffer (100% formamide, 50X Denhardt's, 20X SSC, 10% Tween, and DEPC water) with Block (HBB-Hybridization buffer, 50mg/l yeast tRNA, 100mg/l Heparin and 10mg/l salmon sperm DNA ) for 10 minutes without nutating. The 1:1 PBST: HBB was removed and replaced with HBB. The embryos were incubated for 10 minutes in HBB. Then the embryos were heat treated in hot block with water, at 75°C for 30 minutes. Pre-hybridization was done using a water bath at 65°C for 3 hours.

Probes were removed from -20°C and kept on ice. The probes were diluted in HBB to a final concentration of 1:1000. The probes were denatured by incubating at 80°C for 5 minutes, and then were chilled on ice. Hybridization was done using a 65°C water bath for 16 hours. After 16 hours the embryos and probe were diluted with 500 µl of pre warmed Hybridization Buffer (HB) in the water bath. Embryos were washed with 1000 µl pre warmed HB, for 2-3 hours at 65°C replacing HB every hour. After 3 hours, the embryos were washed for 30 minutes at 60°C in 500 µl of 1:1 HB: 1XPBST. Then the embryos were washed 3X 5 minutes in 1X PBST at room temperature on a nutator.

The hybridized probe was visualized using an antibody stain to the DIG label on the probe using alkaline phosphatase. Embryos were blocked by washing 3X10 minutes in Block solution which was made out of 1XPBST and 2% BSA. Anti-DIG-AP antibody was diluted 1:2000 in Block solution. Then the embryos were incubated overnight in the refrigerator. Antibody was then removed and rinsed with 1X PBST several times. Embryos were washed at least 6X10 minutes in 1X PBST. Then the embryos were washed overnight in 1X PBST in the refrigerator. After washing overnight, embryos were washed in alkaline phosphatase with 0.1%

Tween developing solution for 2X5 minutes. Embryos were transferred into a depression glass. Then the embryos were developed for 3.5-4 hours with 2000 µl developing solution, 9 µl NBT and 7 µl X-phosphate in dark on a nutator. The reaction was terminated by washing well with PBST.

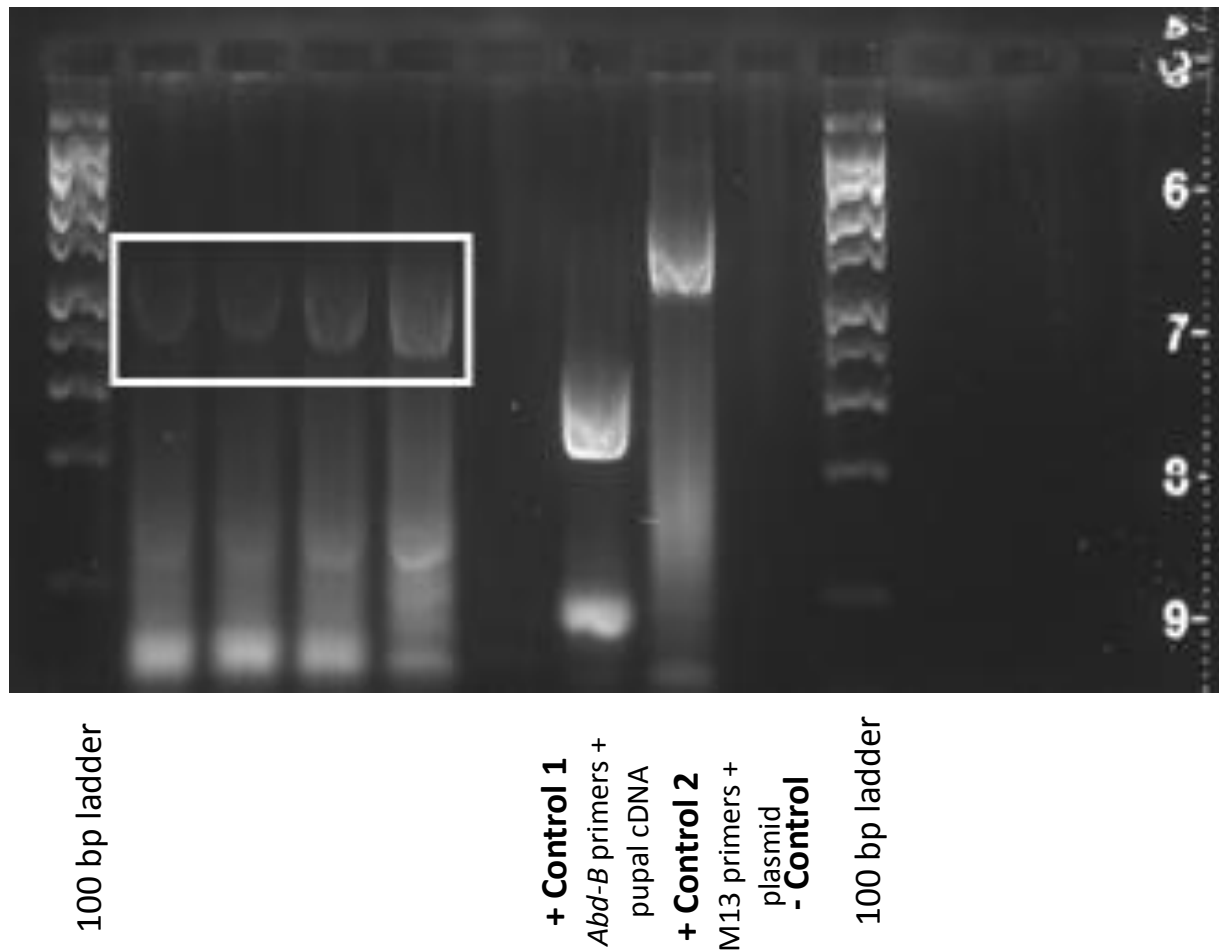
### **Antibody staining for *engrailed***

A secondary antibody stain was conducted with an *engrailed* antibody, which labels the posterior region of each body segment. Embryos were washed on a nutator with 70% MeOH, 29.5% PBT, and 0.5% H<sub>2</sub>O<sub>2</sub> for 20 minutes in order to inactivate endogenous peroxidases. Then the embryos were washed in PBT for 10 minutes. The embryos were incubated in blocking solution for 10 minutes while nutating. Then the embryos were incubated overnight in 4D9 antibody (Patel et al. 1989): blocking solution 1:3. A total of 200 µl was used. Then the embryos were washed 3 times fast in PBT followed by washes with 3 times for 10 minutes. Then the embryos were incubated in goat anti-mouse HRP secondary antibody: blocking solution 2:300 for 5 hours. Next, the embryos were washed 3 times fast in PBT and followed by washes with 3 times for 10 minutes. In order to develop a brown color stain the embryos were moved into a 9 well spot plate with 300 µl DAB. The embryos were incubated in dark for 10 minutes. Then 4 µl of H<sub>2</sub>O<sub>2</sub> was added (2 µl first, and after ~2 minutes, another 2 µl) and mixed gently in DAB. The embryos were developed for ~ 5 minutes. Once the segments are stained, the reaction was stopped by removing DAB, and rinsing with 4 times fast in PBT. The embryos were then stored in 70% glycerol until mounted. Then the embryos were photographed using a Leica DFC295 camera connected to a Leica DM 750 microscope.

## Results

### Obtaining sequence for *Themira biloba* Bithorax genes

A sequence over 750 bp was needed to make a probe for *abd-A*. In order to obtain a longer sequence, a RACE PCR was performed with *T. biloba* pupal cDNA (Figure 3.1).



**Figure 3.1:** RACE PCR amplification of *T. biloba abd-A*. The bands marked with the white square were sequenced and later verified as being *abd-A*. All the selected bands were in the range of 400 bp- 450 bp.

Approximately a 508 bp sequence was obtained from this sequencing. This was combined with the already available sequence, and a long enough sequence with 925 bp was created (Figure 3.2).

```

      20      40      60      80
abdALongest TTTTTTTTTTCTGTTTCGATATTCAACTCTTTGTCGGCCAAGTCTTATCATTTGCCATTCCAGAGTTTCTGATTAAATTCGAGCCCTACAC

      100     120     140     160     180
abdALongest GCACACACACACACACATACCACACCCTTCAACGCAAGCATTTTCCACATTTTATAGAATATCTATTACTTGATGAATCGAAAGTTGAGT

      200     220     240     260     280
abdALongest ATCGAACAACATAATTGAATAATGGTTTGTGTTGCACCTTCTTGTACAGGTCCTCAATGGCTGCCACGACGACGCGGTGCGCCAGACTTACACACGC

      300     320     340     360
abdALongest TTCAAACACTCGAACGGAGAAGGAGTTCCATTTCAATCACTATTTGACGCGGCGGCGAAGAATCGAAATCGCCACGCCCCTATGCCTAACTG

      380     400     420     440     460
abdALongest AGAGACAAATTAAGATTTGGTTCCAGAATCGCCGCATGAAATTGAAAAAGGAGCTTCGAGCCGTCAAAGAAATCAACGAGCAGGTAAGTCAGGT

      480     500     520     540     560
abdALongest CTTCTCACACCGCACACGATCGATCAGCAGCAATTGACCGATCCAAAATCGTAAGATGGAAATTAATGACATGCAATTGTTTCTCTTCACGTT

      580     600     620     640
abdALongest TTGTCCATACCGTATAGGCTAGACGCGATCGCGAAGAACAAGAGAAAATGAAAGCTCAAGAATCCATGAAATCCGCCAGCAAAATGCCAACA

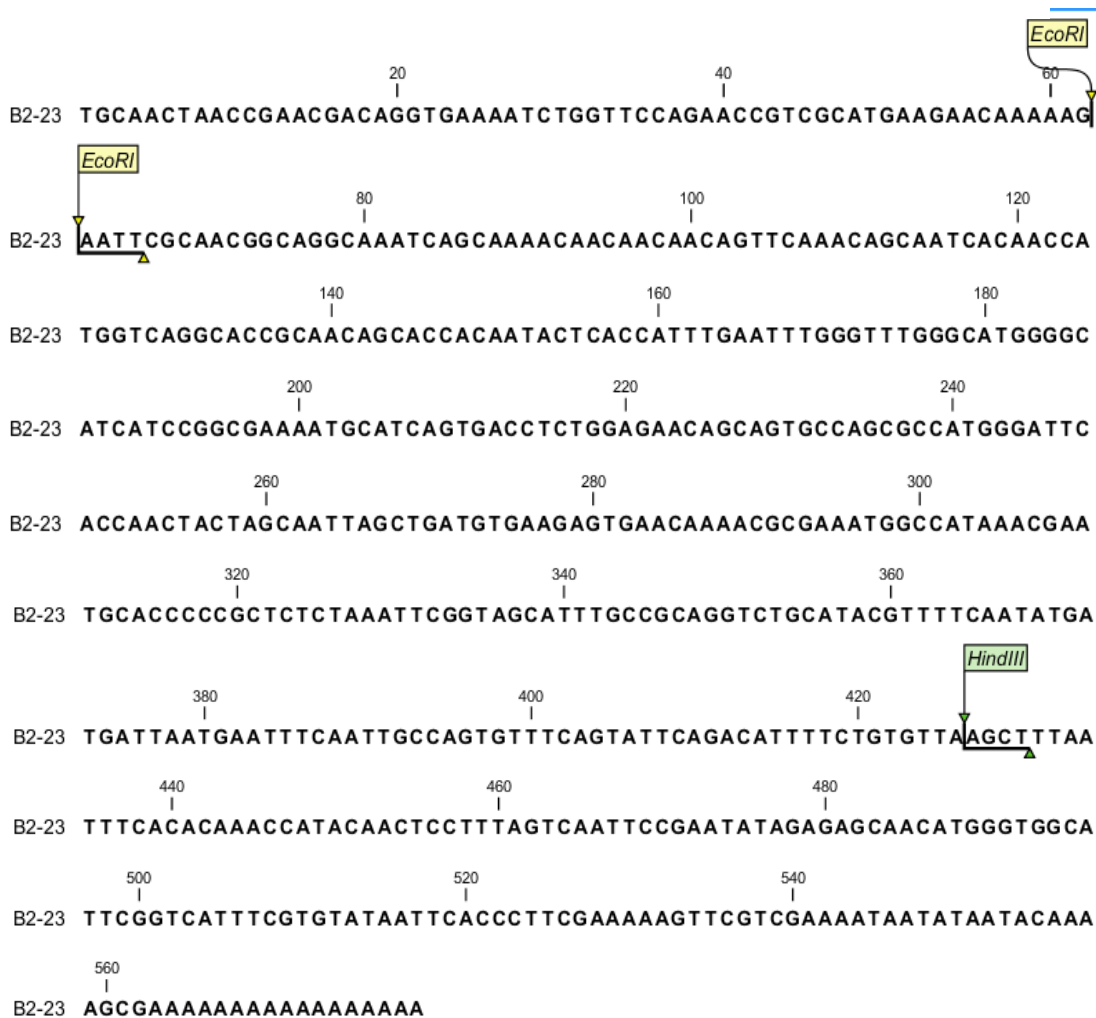
      660     680     700     720     740
abdALongest AGCAAGTGCAACAACAACAGCAACAGCAGCAACAACAGCACCAGCAGCAACAACAACACCAACAACAACAGCAGCAGCAACAGCAACAGCA

      760     780     800     820     840
abdALongest GCAGGATCATCACTCCATCATCGCCATAACACAGGACACTTACATCACTCGGTGGTGGGCCAAAACGATTTGAAACTCGGTTTGGGCATGAGC

      860     880     900     920
abdALongest GCCGGCCTAGGGGCTGGCATTGGCGGCGGCCTGGGCATGATGAGCGCACTCGACAAGACCAACCACGACCTACTGAAGGCA

```

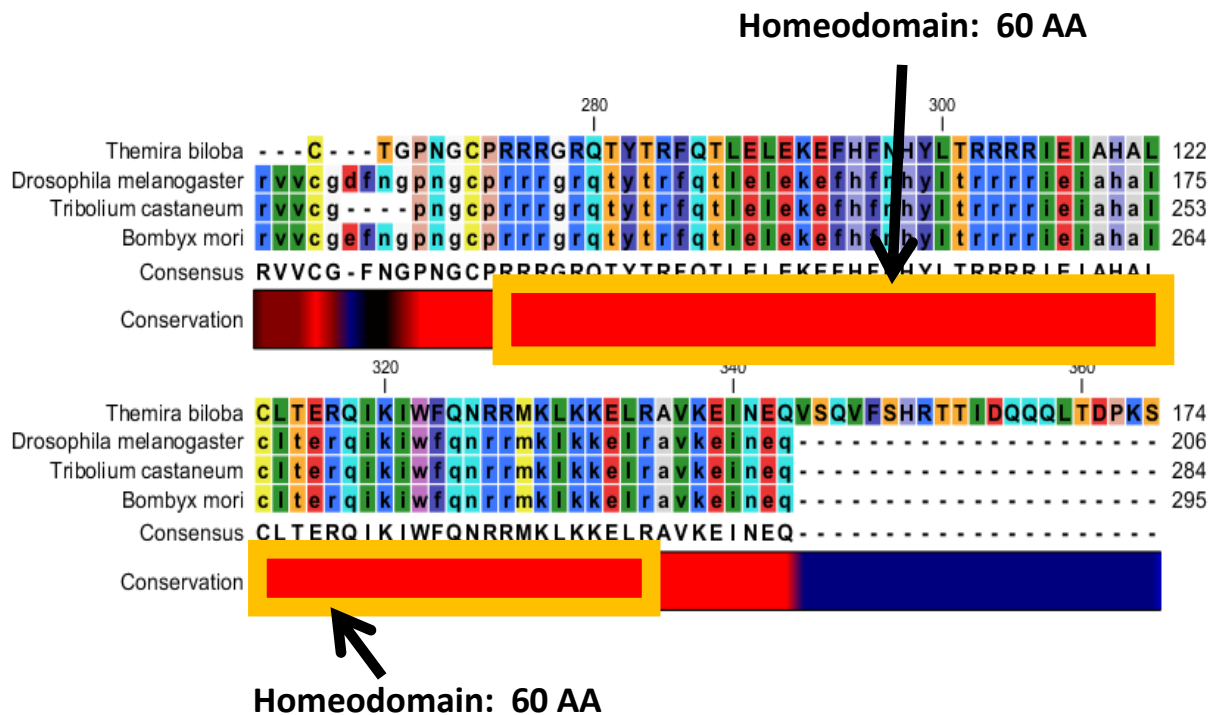
**Figure 3.2:** A partial sequence of *T. biloba* *abd-A*. Multiple sequence fragments were aligned to make a consensus. This sequence was analyzed through alignment and BLAST and was used to make a probe for *in-situ* hybridization.



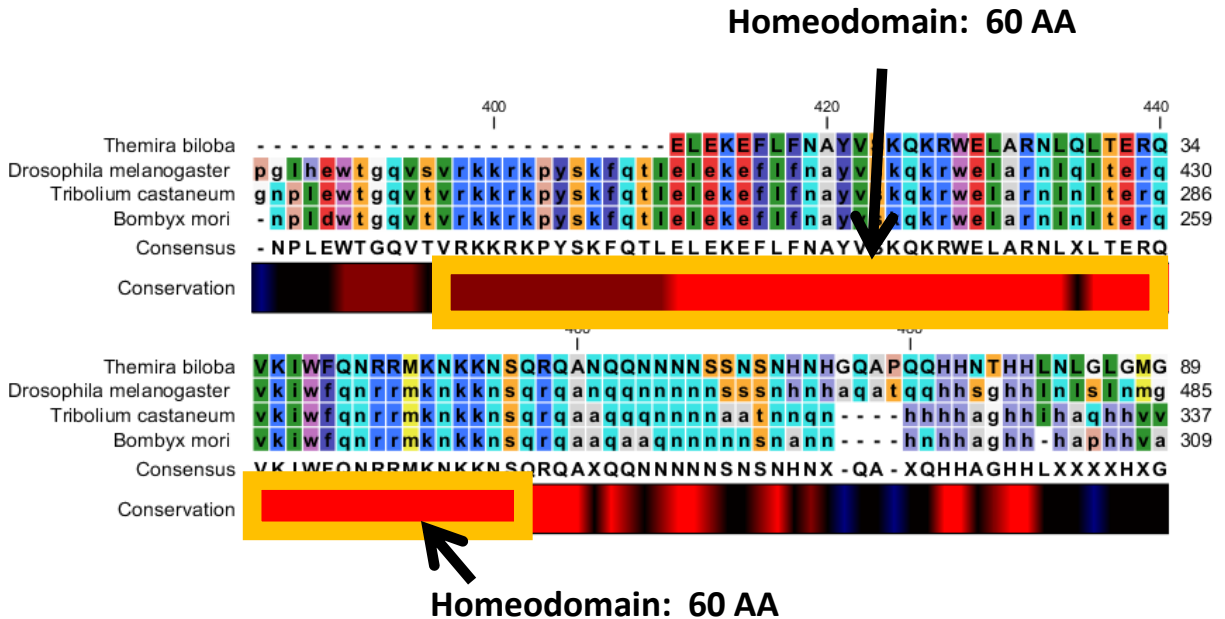
**Figure 3.3:** A partial sequence of *T. biloba* already available *Abd-B*. Multiple sequence fragments were aligned to make a consensus. This sequence was analyzed through alignment and BLAST and was used to make a probe for *in-situ* hybridization (© Julia Bowsher).

The sequence obtained was analyzed in NCBI BLAST program and aligned with *abd-A* of different insect species such as *Drosophila melanogaster* (53%; 3e-65), *Tribolium* (37%; 1e-44), and *Bombyx mori* (36%; 3e-65) (Figure 3.4).

*Abd-B* was also aligned with *Abd-B* of 3 other insect species such as *Drosophila melanogaster*, *Tribolium*, and *Bombyx mori* (Figure 3.5).



**Figure 3.4:** The alignment of sepsid *abdominal-A* with *Drosophila melanogaster*, *Tribolium*, and *Bombyx mori*. The red region shows the conserved region with identical amino acids. Black region is where two amino acids are identical, and the dark red region is where amino acids are identical among three species. The blue region is where none of the amino acids are conserved among all four species. The yellow square represents the Homeodomain.



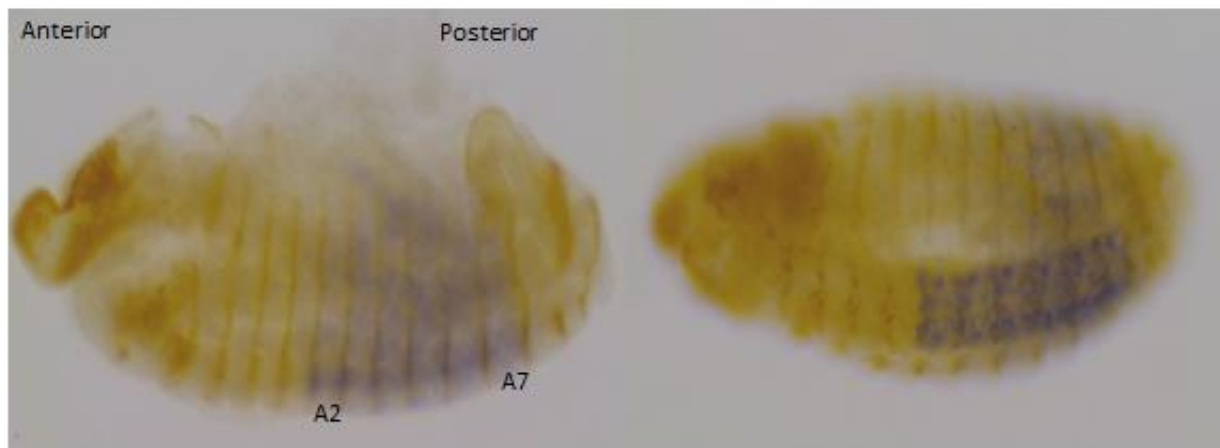
**Figure 3.5:** The alignment of sepsid *Abdominal-B* with *Drosophila melanogaster*, *Tribolium*, and *Bombyx mori*. The red region shows the conserved region with identical amino acids. Black region is where two amino acids are identical, and the dark red region is where amino acids are identical among three species. The blue region is where none of the amino acids are conserved among all four species. The yellow square represents the Homeodomain.

### ***In-situ* hybridization and antibody staining**

In order to identify the expression pattern of gene *abd-A*, *in-situ* hybridization was done for *T. biloba* embryos. The embryos were double labeled for *Engrailed* (brown) with an antibody staining in addition to *abd-A* (blue). Segment boundaries of the embryos were labeled using *engrailed*.

Different stages of embryos were labeled with *in-situ* hybridization and antibody staining, and observed under a Leica DFC295 camera connected to a Leica DM 750 microscope. However, some of them showed no gene expression because *abd-A* is not expressed in early stages of *T. biloba* embryos. The embryos included in here are after the stage of germ band retraction, and are from Stages 12-15. In germ band retraction the germ band retracts so that the

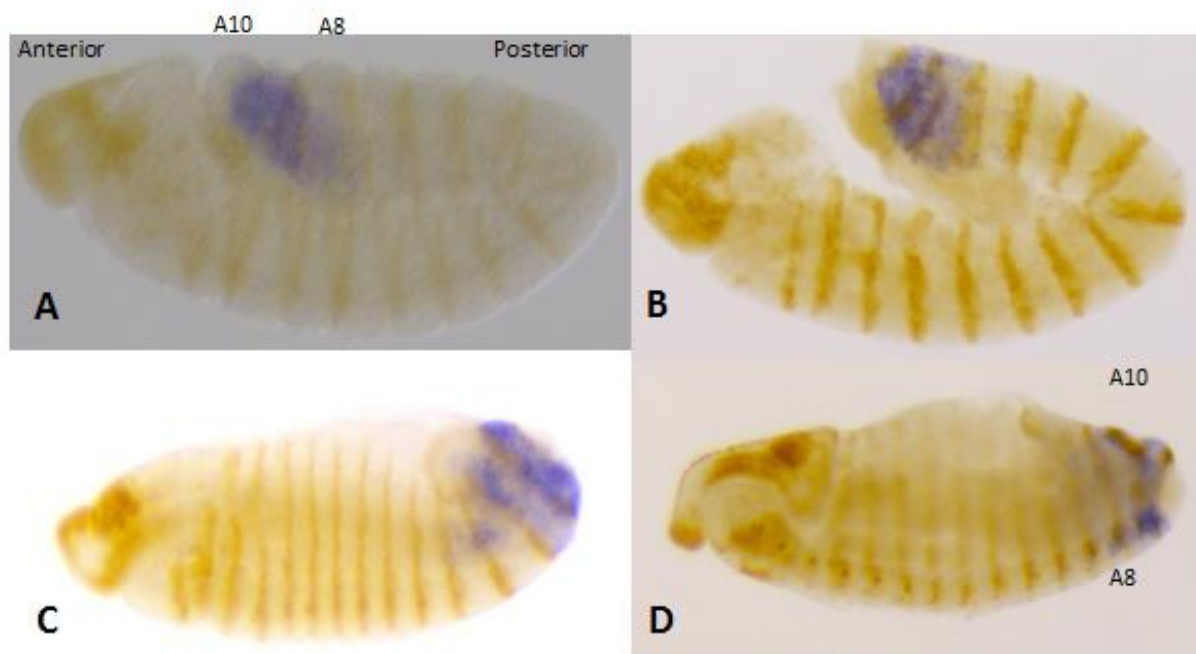
opening of the hindgut comes over to the dorsal side of the posterior egg pole and the width of the germ band increases in almost one and a half time. *abd-A in-situ* hybridized embryos of sepsid *T. biloba* showed an expression pattern from segment A2 –A7 which is from posterior of parasegment 7 to anterior of parasegment 13<sup>th</sup>. Segments are derived from parasegments. Parasegments are also used to describe the expression patterns in embryos. Each parasegment is divided into 2 parts, anterior and posterior. The anterior of a parasegment line-up with the posterior of a segment and the posterior part of the parasegment will line up with the next segment's anterior part. The expression was much darker when viewing from the bottom of the embryos due to the neural ganglia of each segment (Figure 3.6).



**Figure 3.6:** Expression of *abdominal-A* protein in *Themira biloba* embryos after the germ band retraction period. (A) Stage 12-14 embryo lateral view showing the general domain of *abd-A* expression in segments A2-A7 (Parasegment 7-13) for 6 whole segments. (B) Ventral view of Stage 12-14 embryo *abd-A* expression. The expression looks much darker due to the neural ganglia.



*In-situ* hybridization was done to see the expression of *Abdominal-B* protein in *T. biloba* embryos. The *Abd-B in-situ* hybridized (blue) embryos were also double labeled for *engrailed* (brown), with an antibody staining for *engrailed* was used to identify the segment boundaries. The *in-situ* hybridized embryos of sepsid *T. biloba* showed an expression domain from A8 to A10 (Figure 3.7). The expression looks a little faint in the most anterior segments compared to posterior segments.



**Figure 3.7:** Expression of *Abdominal-B* protein in *Themira biloba* embryos after the germ band retraction period. All the embryos are showing the lateral view. (A) Stage 11 embryo showing the general domain of *Abd-B* expression in segments A8-A10 for 3 whole segments (B) Stage 12 embryo showing the general domain of *Abd-B* expression. The segment A10 had the most distinct labeling. (C) Stage 13 embryo with *Abd-B* expression. (D) Stage 14 embryo showing the *Abd-B* expression.

## Discussion

The genes involved in specifying the novel abdominal appendages in *T. biloba* male had not been identified previously. Discovering the genetic regulation behind this fascinating structure is important for further developmental research on *T. biloba*. Due to the role of Hox genes in segment specification, we predicted that one or more bithorax genes are responsible for the process of abdominal appendage development. We hypothesized that the expression domain of one or more bithorax genes may have shifted in order to specify this structure.

In *Drosophila* *abd-A* expression domain is identified from abdominal segment A2-A8 which is parasegment 7-13 (Macias et al. 1990). Our results from the double labeling of *engrailed* and *abd-A* of *T. biloba* stage 12-14 embryos show an expression domain from A2-A8. *Drosophila* embryo is segmented and has 3 main parts. There are 3 head segment, 3 thorax segments and 9 abdominal segments, and this number of segments is the same in *T. biloba*. Therefore, the gene *abd-A* expression domain in *T. biloba* is the same as in *Drosophila*.

In *Drosophila*, there are two *Abd-B* transcripts which encode two protein isoforms; *Abd-Bm* and *Abd-Br*. Their domains are not overlapping with each other, and they have different functions (Clenicker et al. 1990). The *Abd-Bm* protein works with *Ubx* and *abd-A* to pattern the abdomen, while the *Abd-Br* protein acts to specify the reduction of segments at the end of the abdomen. Additionally the *Abd-Br* protein suppresses *m* protein function eliminating segments of the embryo (Casanova et al. 1986, Celnicker et al. 1990, Kuhn et al. 1992, Estrada and Sanchez-Herrero 2001, Yoder and Carroll 2006). Furthermore, the *Abd-Br* protein is thought to suppress segmentation in *Drosophila*, by repressing the expression of *Ubx* and *abd-A* in addition to the suppression of *Abd-B m* protein (Macias et al. 1990, Kuziora 1993). In addition to the role of *Abd-B* in the posterior abdomen, the *Abd-Br* protein is also needed to specify the genitalia.

Out of the two isoformes, *Abd-Bm* is expressed more anteriorly in the abdomen, from segments A5-A8, which is parasegment 10-13, while the expression domain of *Abd-Br* is expressed towards the posterior from segments A8-A10, which is parasegment 14-15 (Casanova et al. 1986, Celnicker et al. 1990, Kuhn et al. 1992). According to our results, *Abd-B* expression domains in Stage 12 *T. biloba* embryos go from segments A8-A10 which is parasegment 14-15. Therefore, the *in-situ* hybridized embryos of sepsid *T. biloba* show the same expression as *Abd-Br* protein in *Drosophila*.

As stated in our hypothesis, we predicted that the initial signal to the histoblast cells to proliferate into abdominal appendage formation may be controlled by the bithorax genes, and that a change in the normal expression pattern of these genes underlies this proliferation. However, according to our results, we did not observe a change in the expression patterns for *abd-A* and *Abd-B* genes. There are many occurrences in the insect world that a change in various expression domains of Hox genes changed the phenotype of otherwise related organisms. In crustaceans, modification of the anterior thoracic limbs into feeding structures is due to a change in expression patterns of *Ubx* and *abd-A* genes (Averof and Patel 1997). Simcox et al. 1991 determined that the removal of bithorax genes caused an inhibition of the appearance of histoblasts cells and caused imaginal discs to form on the abdomen in *Drosophila*, indicating that bithorax gene are involved in histoblast specification. Further, a small change in the expression pattern of *Ubx* protein resulted a difference in bristle patterns in second thoracic femur in closely related *Drosophila* species (Stern 1998). Even though all these examples show that the change in expression patterns of otherwise conserved genes may be a possibility for a particular change in morphology, our results show that the *abd-A* and *Abd-B* gene expressions are conserved in *T.*

*biloba*. However, the gene *Ultrabithorax*, which is the other gene in the bithorax complex, could be a possible candidate and should be investigated in future studies.

Hox genes encode transcription factors that regulate other genes. Therefore these transcription factors can regulate target genes and form new morphologies (Carroll 1995, Weatherbee et al. 1998, Barmina and Kopp 2007, Hersh et al. 2007). So, even though there is not a shift in the expression pattern of *abd-A* and *Abd-B* genes in *T. biloba*, there is a possibility of the downstream targets to evolve, and this might be responsible for the abdominal appendage formation. Investigations of gene expression cannot test this hypothesis, and a functional analysis is required. For example, if the genes are knocked down singly or in combination, the formation of abdominal appendages could be inhibited, and the abdominal appendages will be lost. Therefore, identifying the function of these genes by knocking down the gene using RNA interference would be a logical next step in this research.

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## CHAPTER 4: GENERAL CONCLUSION

Why do organisms look the way they look? Why do turtles have a shell, why do giraffes have a long neck, why do butterflies have eye spots in their wings, why do snakes not have legs? Organisms show distinct characters compared to each other due to many reasons, and these characteristics have been naturally selected in these organisms.

Due to their nature, novel structures contributed greatly to the diversity among species. They contribute to changes from genes to organisms and finally to the whole ecosystem (Shubin and Marshall 2000). Therefore, understanding the importance of evolutionary novelty and the developmental basis behind an evolutionary novelty is highly important.

My studies on Chapter 2 identify the fitness benefit of these structures to sepsid flies and provide answers for some of the gaps in identifying the importance of this novel structure to sepsid flies. My studies reveal that sepsid *T. biloba* are unable to successfully mate without these novel abdominal appendages. Specifically, the long bristles compared to the short bristles on the abdominal appendages provide an advantage in copulation for *T. biloba*. The exact reason behind selecting long bristles over short bristles is unknown. However, I hypothesize that may be the long bristles are capable of reaching the ventral side of the female abdomen due to their length, and these bristles might help to grasp females in addition to the forelegs of the males that are normally used at copulation. Also the long bristles might be long enough to stimulate the female abdomen by rubbing compared to the short bristles.

The experimental evidence on identifying the gene expression in Chapter 3 shows that the expression domain of *abd-A* and *Abd-B* genes are conserved in *T. biloba* abdomen. Therefore, my hypothesis regarding a gene expression change is not supported for these two genes. The genes which are the basis of a phenotype of an organism are highly conserved among

all bilateral organisms (Abzhanov and Kaufman 1999). Therefore, it cannot be that new genes are responsible for specifying these structures. That is why the idea that the same genes, by changing their expression or changing the downstream targets, might have contributed to the formation of novel appendages in sepsids is plausible. As this study could not identify the expression of *Ubx*, there is a possibility of that gene to be involved in this process by having a change in expression domain. At the same time, even though the expression is conserved, it is possible that the downstream targets of these genes will contribute an answer for the question. Therefore, knocking down genes individually and in combination with RNA interference experiments will be valuable.

In summary, my research findings on sepsid mating confirmed the necessity of abdominal appendages for successful mating in *Themira biloba*. This provides the link for future research on identifying why only long bristles of the novel structure are important in *T. biloba* mating. Additionally discovering that the gene expression is conserved in *T. biloba* provides insights for further research on a different aspect on the genetic regulation of the specification of abdominal appendages of *T. biloba*.

### **Literature cited**

- Abzhanov, A. and Kaufman, T.C. 1999. Novel regulation of the homeotic gene *Scr* associated with a crustacean leg-to-maxilliped appendage transformation. *Development* 126:1121–1128.
- Shubin, N.H., and Marshall, C.R. 2000. Fossils, genes, and the origin of novelty. *Paleobiology Supplement* 26:324–340.

# APPENDIX A: IDENTIFYING THE FUNCTION OF BITHORAX GENES TOWARDS THE FORMATION OF THE NOVEL ABDOMINAL APPENDAGES IN

## *THEMIRA BILOBA*

### Background

The three bithorax genes, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) may be responsible for the specification of the abdominal appendages in male sepsid flies. In chapter 3, I hypothesized that there can be a shift in the gene expression of the bithorax genes which can be responsible for forming the novel abdominal appendages in the 4<sup>th</sup> abdominal segment in sepsid *Themira biloba*. However, as concluded in chapter 3, the bithorax genes *abd-A* and *Abd-B* are conserved in *Themira biloba* compared to other insects. Therefore a shift in the expression pattern of *abd-A* and *Abd-B* has not contributed to the specification of the 4<sup>th</sup> abdominal segment appendages in *T. biloba*. On the other hand, these genes encode transcription factors. Transcription factors regulate the expression of particular genes by binding with other transcription factors. So, even though a shift in the expression pattern for *abd-A* and *Abd-B* has not occurred in *T. biloba*, I predict that knocking down one of the bithorax genes will inhibit the formation of abdominal appendages in *T. biloba*.

### Methods

To examine the function of each bithorax gene, I conducted loss-of-function experiments by knocking down the Bithorax function with RNA interference (RNAi). RNAi is a process in which long double stranded RNAs (dsRNA) specifically suppress the expression of a target gene. I injected dsRNA into *T. biloba* embryos and as well as 3<sup>rd</sup> instar larvae for the 2 bithorax genes

*abd-A* and *Abd-B*. As a second attempt, I injected Stealth RNA into *T. biloba* embryos and 3<sup>rd</sup> instar larvae.

### **dsRNA synthesis**

The dsRNA were synthesized using the MEGAscript T7 kit (Ambion). As T7 RNA polymerase was produced in a single strand RNA, I prepared one DNA template with opposing T7 promoters and used it in a single transcription reaction in order to synthesize dsRNA. T7 promoter sequence was added to DNA with the use of PCR. The PCR primers were synthesized with the T7 promoter sequence appended at 5' end. The primers for each gene were designed using Primer 3 software. Primers used for each gene was as follows:

abdAFT7 - TAATACGACTCACTATAGGGAGACATCATCGCCATAACACAG

abdART7 - TAATACGACTCACTATAGGGAGAAGGTCGTGGTTGGTCTTGTC

AbdBT7F -TAATACGACTCACTATAGGGGCTATGGGATTCACCAACTA

AbdBT7R - TAATACGACTCACTATAGGGCGAACTTTTTCGAAGGGTGA

UBXT7F2 - TAATACGACTCACTATAGGGAGCATGTTGCATTTCGACAAG

UBXT7R3 - TAATACGACTCACTATAGGGCGAGAAGCCCTTGAACAAAA

The PCR products were then visualized with gel electrophoresis to see whether the products are of the expected size. The DNA was purified in order to have a greater yield of dsRNA. The transcription reaction was done at room temperature and incubate for 4 hours at 37°C. the reaction mixtures were stored in the -20°C for a day. Then 30 µl of nuclease free water and 30 µl LiCl were added. Then the mixtures were mixed well and chilled for 35 minutes.at -

20°C. then the samples were centrifuged at 4°C for 15 minutes at maximum speed. Left the samples overnight in the freezer at -20°C. The samples were re-centrifuged at 4°C. The supernatant was removed and 1 ml 70% RNase free ethanol was added. Then the samples were re-centrifuged at 4°C for 15 minutes at maximum speed. The ethanol was removed and re-suspended in 40 µl of RNase free injection buffer. The dsRNAs were stored at -20°C until used for injection.

### **Embryonic injections**

Embryos were collected within 2 hours after a new defrosted dish is placed in a fly container. The syncytial stage of the fertilized embryos lasts from the time an egg is fertilized until approximately 2 hours later. This is the best time for gene silencing injections as nuclei do not have cell membranes. Additionally germ band elongation starts at 3.5 hours and goes until 4.5 hours. This is the time period when *Hox* gene expression starts. These are the reasons for selecting embryos that are no older than 2 hours.

The eggs were washed with the egg wash solution using a mesh basket. The embryos were dechorinated with the use of a double sided sticky tape and dechorinated embryos were placed on a cover slip which is placed on a slide with the posterior end of the embryo towards the close edge of the cover slip. Glue made out of double sided tape (Scotch brand) in n-Heptane was used to keep the embryos without moving on the cover slip. A halocarbon oil was used to keep the embryos away from drying out and also helped the embryos heal injection sites.

Needles for injections were made out of glass capillary tubes with a verticle needle puller. The needle that was used in the injection was back-loaded with dsRNA of either *abd-A*, *Abd-B*, or *Ubx*. dsRNAs were combined with a tracer dye in order to visualize the injection through microscope (dye:dsRNA 1:10). The needle was lined up and a joystick will be used to

control small movements. The needle was broken using razor blades or by touching the cover slip edge. A 1.2-1.8 PSI injection and a 0.4-0.6 PSI balance was used when injecting. As a control, a saline buffer which was also used in diluting the dsRNA was injected to a number of embryos each time. Injections were done just inside the cell membrane without making a significant damage to the embryo. The injected embryos were placed in a moist petri dish for 5 hours, and then extracted RNA in order to perform semi-quantitative PCR.

### **Injections of third instar larvae**

Injections were also done for the 3<sup>rd</sup> instar larvae of *T. biloba* to see any deformities due to dsRNA during the embryogenesis. For this, 3<sup>rd</sup> instar larvae were collected and had them wander on moist filter paper to clean their outside before pupation. Once the larvae has stopped moving, but before the puparium had fully melanized, they were placed on a glass slide with double sided tape to keep them in one place. Puparia were injected following the same procedure as the embryos. After injection, the puparia were kept in properly moistened petri dishes for 5 hours and extracted RNA for semi-quantitative PCR, or they were allowed to develop to adults.

### **Semi-quantitative RT-PCR**

In order to extract the RNA, 10 embryos were added in one 1.5 µl microcentrifuge (blue) tube. When extracting RNA from injected 3<sup>rd</sup> instar larvae (now pupae), only one larvae were inserted in 1 tube. Then 1000 µl was added in each tube in 2 steps. First, a 50 µl was added to each tube. The embryos were homogenized with pestals and the rest of the 950 µl of Trizol was added. The embryos were incubated for 5 minutes and then 200ml of Chloroform was added. The tubes were inverted multiple times and incubated at room temperature for 2-3 minutes. After

centrifuging at 12,000rpm for 15 minutes, the aqueous layer was removed and 500 µl of isopropyl was added. After a 10 minute incubation, the samples were centrifuged for 15 minutes. The supernatant was removed and the pellet was washed with 75% EtOH in DEPC treated water. Then the samples were vortexed and centrifuged for 5 minutes. As much as alcohol was removed from tube and the pellet was resuspended with DEPC-treated water. Then it will be stored in the freezer overnight.

Promega cDNA synthesis kit, (GoScript Reverse Transcription System) was used to synthesize cDNA. The cDNA was used to do the semi-quantitative PCR in order to see for evidence in gene silencing. Hox gene specific primers respectively abdAF1, abdAR1 for dsabdA injected embryos or larvae; qPCRAbdBf and qPCRAbdBR for dsAbdB injected embryos or larvae; and qPCRUbxF and qPCRUbxR for dsUbx injected embryos or larvae were used in the semi-quantitative PCR.  $\beta$ Actin primers ( $\beta$ actin 2F and  $\beta$ actin 2R) were used as a reference gene amplification.

The primer sequences are as follows:

abdAF1 -CATCATCGCCCATAACACAG

abdAR1 - GTGGTTGGTCTTGTCGAGTG

PCRAbdBf - CCATTTGAATTTGGGTTTGG

qPCRAbdBR - CGCGTTTTGTTCACTCTTCA

qPCRUbxF - AGCTGAACGAGCAGGAGAAG

qPCRUbxR – TTGTAGTTTGTGGCCAGTCG

$\beta$ Actin-2F - ACCAATTGAGCACGGTATC

$\beta$ Actin-2R - GGTGAGCAAGACTGGGTGTT

## **Stealth RNA injections**

Stealth RNA was synthesized by a company according to the same sequences as used in dsRNA.

The sequences for the Stealth RNAs were as follows:

*abd-A*- GACGAUCGCUGUAGCGGUUACAGUA

*Abd-B*- UACUGUAACCGCUACAGCGAUCGUC

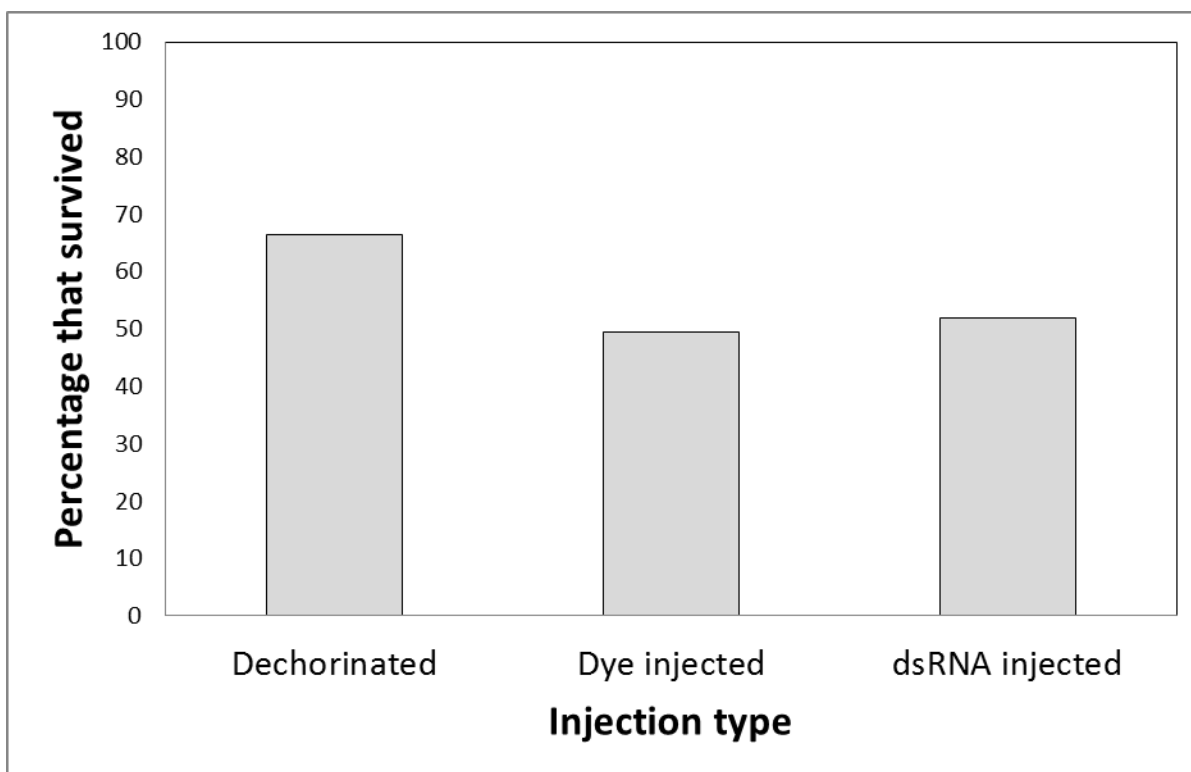
The procedure used for Stealth RNA injections was same as dsRNA injections. In addition to running a semi-quantitative PCR with the cDNA from injected embryos and 3<sup>rd</sup> instar larvae, they were raised on cow dung in order to observe the phenotype.

## **Results**

### **Survivorship of the embryos after injections**

The percentage of the embryos survived were graphed for dechorinated, dye injected, and dsRNA injected embryos (Figure A.1). According to the observation of the hatching rates, in total 210 dechorinated only embryos, 350 dye injected embryos, and 160 dsRNA data are included in the graph.

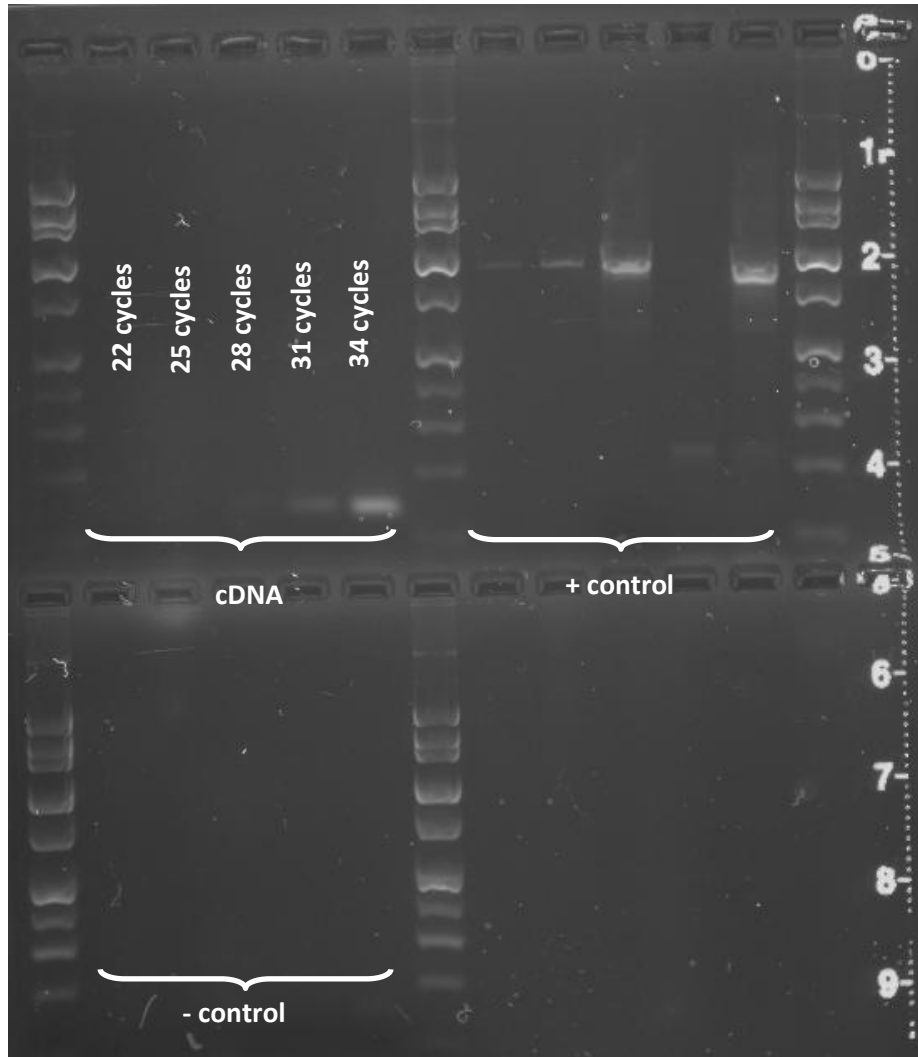




**Figure A.1:** Survival rate of the embryos which are dechorinated only, dye injected, and dsRNA injected.

### **Deciding the number of cycles for Semi-quantitative PCR**

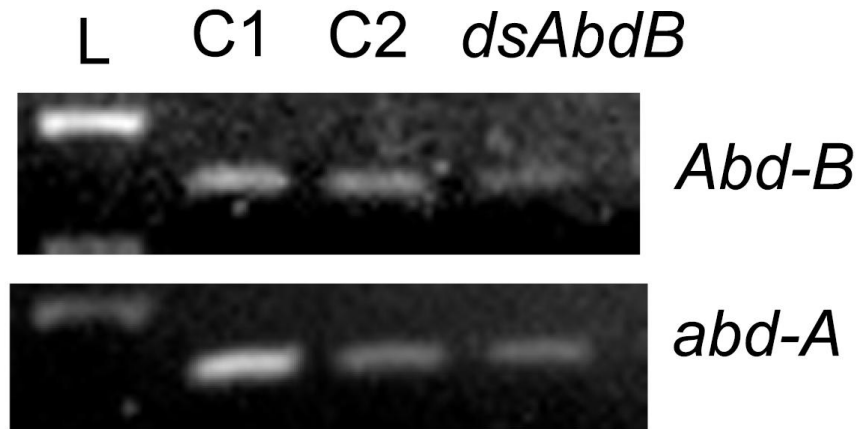
I also performed an optimization experiment to obtain the number of cycles to be followed in Semi-quantitative Reverse Transcription PCR. Five cycles were tested starting at 22 cycles and ending at 34 cycles with an interval of 3 cycles. This test was done for the experimental group (with cDNA) the positive control, and the negative control (Figure A.2).



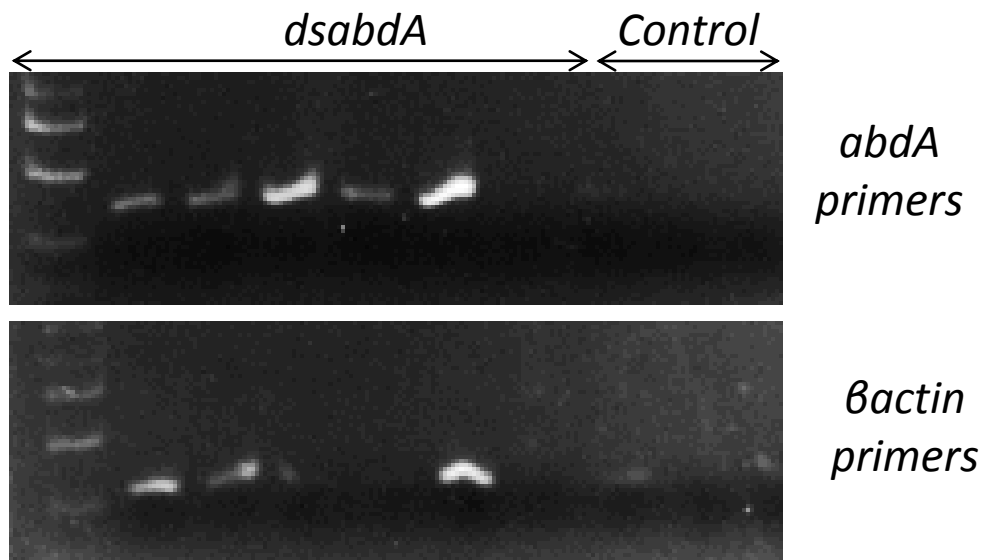
**Figure A.2:** The gel picture of the experiment conducted to optimize the number of cycles to be used in semi-quantitative PCR.

### Semi-quantitative PCR results

Semi quantitative PCRs using the cDNA from the dsRNA and control injected embryos and 3<sup>rd</sup> instar larvae were done. Each PCR reaction with embryonic cDNA had 10 embryos in it, and if it is 3<sup>rd</sup> instar larvae cDNA each reaction had only one pupae in it. However, I have been unable to demonstrate a knockdown in gene expression (Figure A.3, A.4).

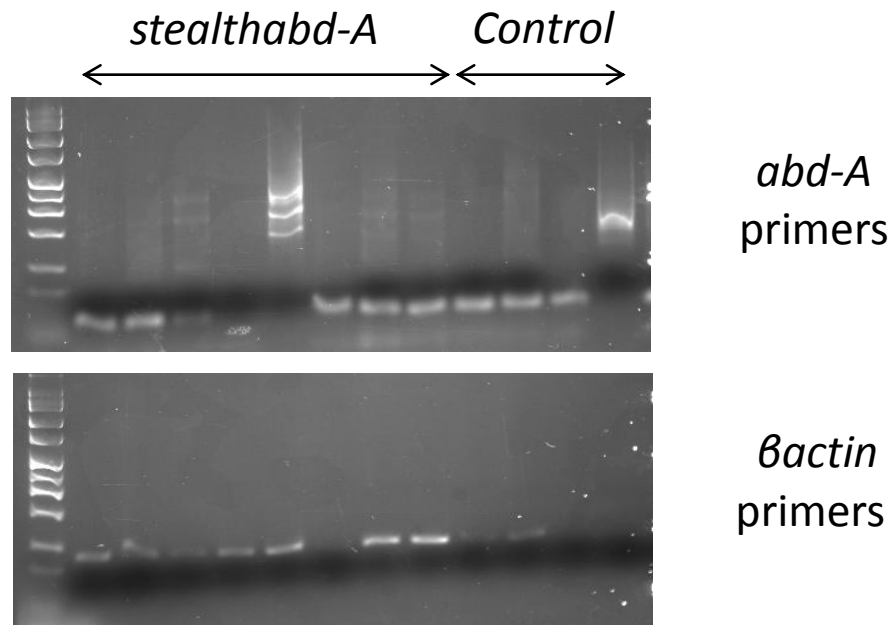


**Figure A.3:** Semi-quantitative PCR of *dsAbd-B* injected embryos. Represents the control injected cDNA and *dsAbdB* injected embryo cDNA amplified with *Abd-B* primers, and *abd-A* primers (as a control). It was expected that if the gene *Abd-B* was knocked down, there will not be any amplification for *dsAbdB* cDNA with *Abd-B* primers. However, there will be an amplification of *dsAbdB* cDNA for *abd-A* primers, and control injections for both primers.



**Figure A.4:** Semi-quantitative PCR of *dsabd-A* injected embryos. Shows the control injected cDNA and *dsabdA* injected embryo cDNA amplification with *abd-A* primers, and  $\beta$ actin primers (as a reference). It was expected that if the gene *abd-A* was knocked down, there will not be any amplification for *dsabdA* cDNA with *abd-A* primers. An amplification of *dsabdA* cDNA for  $\beta$ actin primers (as it works as a reference) and control injections for both primers were expected.

Semi-quantitative PCR was done to analyze gene silencing with stealth RNA injections (Figure A.5).



**Figure A.5:** Semi-quantitative PCR of *stealthabd-A* injected pupa. Shows the control injected cDNA and *stealthabd-A* injected pupal cDNA amplification with *abd-A* primers, and *βactin* primers (as a reference). It was expected that if the gene *abd-A* was knocked down, there will not be any amplification for *stealthabd-A* cDNA with *abd-A* primers. However, it was expected that there will be an amplification of *stealthabd-A* cDNA for *βactin* primers (as it works as a reference), and control injections for both primers.

In addition to the semi quantitative results, none of the stealthRNA injected embryos nor pupae survived on cow dung in raising to adulthood. Dung plates were discarded due to the growth of mold.

Gene silencing was not observed among dsRNA and stealth RNA injections of both embryos and pupae. Sometimes the control injected embryos looked like they were not survived according to the negative *βactin* amplification of control injected embryos and pupae. The gene silencing did not show a consistency among different injection attempts. Therefore in summary I would suggest that gene silencing was ambiguous among both dsRNA and stealth RNA injections for both embryos and larvae and in conclusive.

## **APPENDIX B: IDENTIFYING THE INITIAL SPECIFICATION OF THE NOVEL ABDOMINAL APPENDAGES OF *THEMIRA BILOBA*- HISTOBLAST CELL COUNTS**

### **Background**

In *T. biloba*, the abdominal appendages develop from histoblast cells (Bowsher and Nijhout 2007). These cells are imaginal cells that form the abdominal epidermis, and do not differentiate during embryogenesis like other cells that will form larval tissues. During metamorphosis, histoblasts proliferate to form the adult epidermis. At the larval stage, the histoblasts are undifferentiated. Histoblast cells can be distinguished visually from the polyploid larval epidermal cells which are adjacent (Madhavan and Madhavan 2004), because histoblasts remain diploid while the surrounding cells endoreplicate into polyploid cells by the end of embryonic period. The diploidy of imaginal cells is maintained by the *escargot* gene in *Drosophila*, which is expressed in imaginal cells throughout embryogenesis and the larval period (Hayashi 1993).

The abdominal appendages in *T. biloba* develop from the ventral histoblast nest on the fourth abdominal segment (Bowsher and Nijhout 2007). At the end of the third larval instar, the number of histoblast cells in the fourth ventral histoblast nest of males is twice the number of histoblast cells in the other segments. The number of histoblast cells is constant in females across all abdominal segments (Bowsher and Nijhout 2007). Since the number of histoblast cells can be a sign for the timing of the initial specification of abdominal appendages in *T. biloba*, assaying the number of cells in a nest can be used to determine the initial time point of appendage specification. Therefore, I proposed to label the histoblast cells of the embryo using *in-situ* hybridization of the *escargot* gene so that I could count the histoblast cells in the fourth

abdominal segment. I hypothesized that the number of cells would be elevated in the fourth abdominal segment because the identity of the histoblast cells is specified at this time.

## **Methods**

### **Obtaining sequence for *Themira biloba escargot* gene**

A sequence for *escargot* gene was obtained from the transcriptome of *Themira biloba* generated by the lab (© Dacotah Melicher), gene specific primers were designed using the Primer 3 software.

The sequences of the primers used were as follows:

BHesgF1: TGATCAACGTGAGCGACTGT

BHesgF2: AGGATGAGGGAGTCGATGTC

BHesgR1: AGTGCGTTTGCAGATGAGC

After amplifying and cloning the cDNA using the TOPO cloning and transformation protocol, positive clones were sequenced with T7 and T3 primers. The sequence obtained was blasted using NCBI BLAST program to make sure the correct *gene* was sequenced, and aligned with *escargot* of *Drosophila melanogaster*.

### ***In-situ* hybridization**

The sequence obtained for *escargot* was used to make a probe for *escargot* using DIG labeled RNA probe synthesis protocol. Using a colony PCR, the required template was amplified, and then was analyzed on a gel. A confirmation was done that the gel band was the same size as the cloned fragment, and then the product was purified. Then the product was quantified using Nano drop. Then the DIG labeling reaction was set up with template, DIG NTP, RNasin, 10X buffer, polymerase (either T3 or T7), and H<sub>2</sub>O. All the solutions were kept RNase

free. The reaction mixes were incubated at 37°C for 120 minutes. 1/10 volume of 4M LiCl was added to the reaction mix followed by a three times volume of ice cold EtOH. Next the reaction mixes were incubated at 20°C at 120 minutes, and were spun at 12000xg for 15 minutes at 4°C. The pellet was washed with 70% ice cold EtOH in DEPC treated water. Then again the samples were spun for 5 minutes at 4°C and briefly let 5 minutes to dry. The pellet was re-suspended in 50ul hybridization buffer and stores at -20°C.

*escargot* probe was used to assay the expression of *escargot* gene using *in-situ* hybridization. Embryos laid over an 18 hour window were rinsed several times with egg wash solution, then placed in 50% bleach and egg wash solution for 1 minute to dechorinate the chorion of the eggs. Then embryos were rinsed well with egg wash solution to remove any bleach. The embryos were transferred into a glass scintillation vial with 1:1 solution of heptane: PEM-FA fixative (3.7% FA). Then they were agitated vigorously for 20 minutes on a rotator. The embryos were removed from the fix- heptane interface with a pasture pipet, and placed in a new scintillation vial. An equal amount of 100% methanol was added to n-heptane and shaken vigorously to crack the vitelline membrane. Embryos were washed multiple times in methanol to remove traces of heptane. Embryos were stored in methanol at -20°C.

Prior to hybridization, embryos were pretreated to increase permeability. Embryos were rehydrated from methanol into PBST. A 7:3, 1:1, and 3:7 MeOH: PBST washes were done for 5 minutes, and a 100% PBST wash was done 6 times 5 minutes each time. Then the embryos were treated with non-predigested Proteinase K (1:1000 dilution with PBST). Then a 2 minute PBST wash followed by 2 times 5 minutes washes were done. The embryos were re-fixed in 4% paraformaldehyde/ 1X PBS for 20 minutes while shaking. The re-fixed embryos were washed 5 times 5 minutes. While these washes were taking place, embryos were separated into individual

1.5 ml eppendorf tubes for hybridization with different probes. Embryos were equilibrated in 1:1 PBST: Hybridization Buffer (100% formamide, 50X Denhardt's, 20XSSC, 10% Tween, and DEPC water) with Block (HBB-Hybridization buffer, 50mg/l yeast tRNA, 100mg/l Heparin and 10mg/l salmon sperm DNA ) for 10 minutes without nutating. The 1:1 PBST: HBB was removed and, replaced with HBB. The embryos were incubated for 10 minutes in HBB. Then the embryos were heat treated in hot block with water, at 75°C for 30 min. Pre-hybridization was done using a water bath at 65°C for 3 hours.

Probes were removed from -20°C and kept on ice. The probes were diluted in HBB to a final concentration of 1:1000. The probes were denatured by incubating at 80°C for 5 minutes, and then were chilled on ice. Hybridization was done using a 65°C water bath for 16 hours. After 16 hours the embryos and probe were diluted with 500 µl of pre warmed Hybridization Buffer (HB) in the water bath. Embryos were washed with 1000 µl pre warmed HB, for 2-3 hours at 65°C replacing HB every hour. After the 3 hours the embryos were washed for 30 minutes at 60°C in 500 µl of 1:1 HB: 1XPBST. Then the embryos were washed 3 times 5 minutes in 1XPBST at room temperature on a nutator.

The hybridized probe was visualized using an antibody stain to the DIG label on the probe using alkaline phosphatase. Embryos were blocked by washing 3 times 10 minutes in Block solution which was made out of 1XPBST and 2% BSA. Anti-DIG-AP antibody was diluted 1:2000 in Block solution. Then the embryos were incubated overnight in the refrigerator. Antibody was then removed and rinsed with 1XPBST several times. Embryos were washed at least 6 times 10 minutes in 1XPBST. Then the embryos were washed overnight in 1XPBST in the refrigerator. After washing overnight, embryos were washed in alkaline phosphatase with 0.1% Tween developing solution for 2 times 5 minutes. Embryos were transferred into a depression

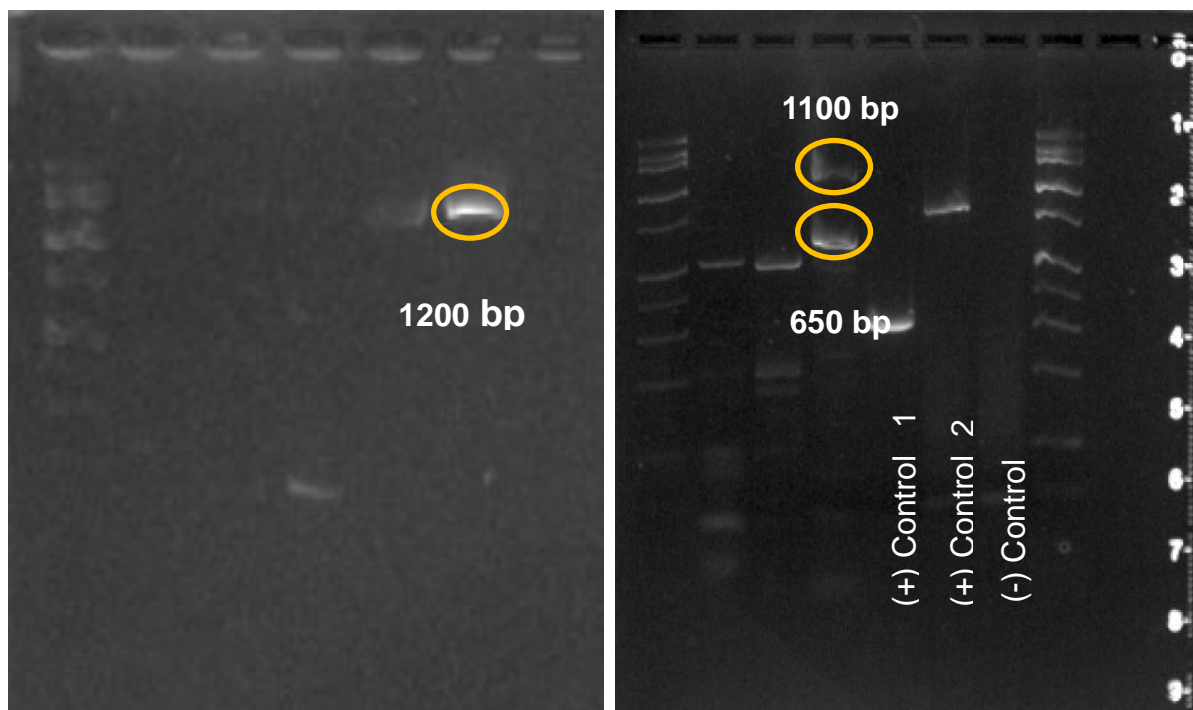


glass. Then the embryos were developed for 3.5-4 hours with 2000  $\mu$ l developing solution, 9  $\mu$ l NBT and 7  $\mu$ l X-phosphate in dark on a nutator. The reaction was terminated by washing well with PBST.

## Results

### Obtaining sequence for *Themira biloba escargot* gene

A RACE PCR was done using the *escargot* gene specific primers and *T. biloba* cDNA. The PCR products were ran on a 2% Agarose gel. The bands marked with a yellow circle were used in sequencing *escargot* gene (Figure B.1).



**Figure B.1:** RACE PCR amplification of *T. biloba escargot*. The bands marked with yellow circles were sequenced and later verified as being *escargot*. (+) Control 1 was *Abd-B* primers and pupal cDNA. (+) Control 2 was M13 Forward and Reverse primers and B2-23 miniprep plasmid. (-) Control was M13 Forward and Reverse primers without DNA template.

The sequence obtained for *escargot* was as follows (Figure B.2.).

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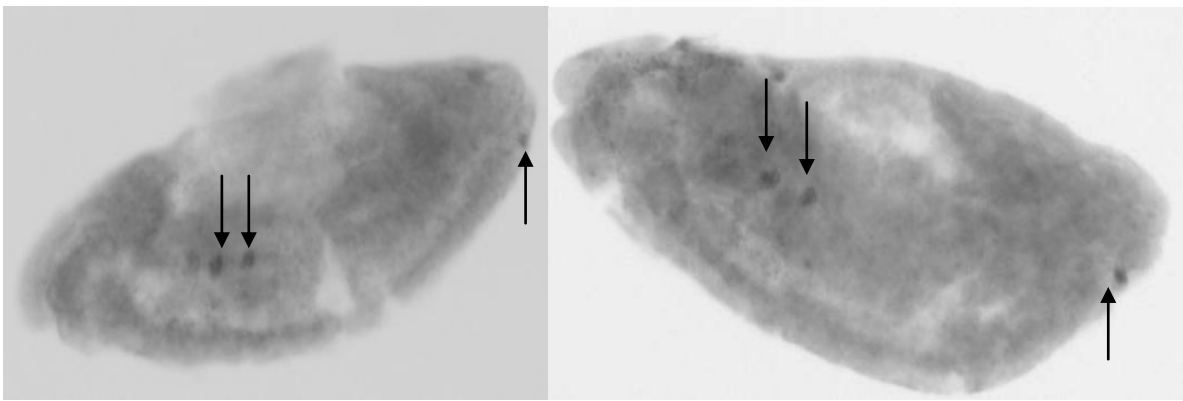
esg  GGGCTTCTCGCCGGTGTGCGTGCGTATGTGGCCTTGCAGCAGCCACGGACGCGAGAATGCCTTGCCGCA
      20      40      60
esg  AATGTTGCATTTTGAGGGCAGCGTATGCGTGCGGATGTGCATTTTGAGAGCGCCCAGCGACACGTACGT
      80     100     120
esg  TTTGTGCGCAGTCCTTGCGAGCTGAACGATTTCTTCACTTGATTACCCCTCGGCGGCGGGCAATGGAAGT
     140     160     180     200
esg  CTGGTGCTTGGTGAGGGCCGGAGAATGTCGAATAGGATTTCTGACAGTCGGGGCATTGGTAGCGCGGCGG
     220     240     260
esg  CGCCGATGATTTTGCTCTTTTGGTGGGCGACGACGTTGAGCAGGTATTGCCACTGCCGCTGCTGTTTGC
     280     300     320     340
esg  GGTGTTGGTGTAGTGTGGTGGTGGTGCAGCAGTGTTCGCCTCGTTTCTCCGACAGACTCATTGCTCTT
     360     380     400
esg  CCTGGCGGCGTCGGCGGTGTGCTTGACGGACAAATCTTCAGGCAGCGCCGCCGAGCAACTCGATTGCGG
     420     440     460     480
esg  CGTCAGCGAAGCATGGAATAGAACGACGACTCGGAGTAGGCCGGTGAGATGGGCGCATGATGGCTGAT
     500     520     540
esg  CGGATGCGGGTGCAACGCATGATGGGGCGCCTGTGGGTGATGCGGCAGCGTTGTGTGATGCGGTGTGAT
     560     580     600     620
esg  GGTGGCCGTGTGATAGCCAGCGGATAGGCTGGCGTCGGCGTGAGGGCGGCATGCGCGTGGCGCATGGTA
     640     660     680
esg  GGCGGCTGCCGAGGGCAGCGCGTACGGCATGAAGCGGTGGTGTGTGTGTGTGGCGGCGCCGGTGCTTTG
     700     720     740
esg  CTGTTGATGGGGGGCAGCGCACTCCGATCGGCGGCGGTGTATGATCTCGGAACGCATGGGCGGCGACACGGA
     760     780     800     820
esg  GCTCGCACGGCCGTGTCAGGTAATGCGGTGGCGGCGACAACGAACCAATCGAATCGGACGGCGAGCTGGG
     840     860     880
esg  CGACAGCAGATCCCCACGACGCGGCGATATTTGCTGCTGCCCCAGATGCGGATTGCTGATGGTGGGATA
     900     920     940     960
esg  GAATTGCGGCGGTGTACGGTGACATGTGGAAGTTCACGGTTGCAACGCGTACTGGGGTATGTGGGCGT
      EcoRI 980     1,000     1,020
esg  GGGCACACCACCGAGGCGGCAGCTGCTGCCGATGCGGCGGCCACTGCTGCAAGCGGCTGCCAGTGCGGC
      1,040     1,060     1,080     1,100
esg  GGCTTGTTGTTGGTGTTCGGAGCATCCGTGTGATTCTCCTCGATGTCCACACAATCGATCATCTCGTCGTC
      1,120     1,140     1,160
esg  GTCGTCATCGTCGTGATCGTGCGAGTGGTCTCGTCATCGCAGCAATCCTCCTCGTCGTGATGTCGGT
      1,180     1,200     1,220     1,240
esg  GGTGTGATCGACATCGACTCCCTCATCTCGCAACAGTCGCTCACGTTGATCAACTCCTCGGAGGAGAC
      1,260     1,280     1,300
esg  TGTGCAATGCGACGAGCTGATTGCCGAATCGCATGAGGATTTCTTGAGGCTGAGATTTTGGGGCTGCGG
      1,320     1,340     1,360     1,380
esg  ATCGCATTGATGTGCTTGGTTCGCAGACGCCTCGTCGAACGATAGGTCACGGGGCGTTTCTTCAAAGG
      1,400     1,420     1,440
esg  ACACTTGCTGTAGTTCTTTTCCACTAACATATCCTCCACGGAATGCATTTTGTGGTTTTAAACAATATT
      1,460     1,480     1,500
esg  CTCTTTATGTTACAAGCAAGAATTGCTTTTTTTTTCAGTTTTATTTGAAATTTTCACTTTTAGAAAATAT
      1,520     1,540     1,560     1,580
esg  TTTTATTGATCTTCACACTTCACTTTGAATGTTTATATTTTCTTATGGGTTTCTTTAGTTGTATTGTAT
      1,600     1,620     1,640
esg  TGTATTGATTATCGATGATCACGCGAAACAATAACAGTAAACTCAATTGTTGTGAACAAAAGAAACAAA
      1,660     1,680     1,700     1,720
esg  CGTTGTGTGATGGAAGCGAGCGAGTGCAGTGCAGAAAGCAAAACGTCTGAACGCAAGCG
      1,740     1,760     1,780

```

**Figure B.2:** The whole sequence obtained for *escargot* for *T. biloba*.



*T. biloba*, and it is possible that the absence of *escargot* expression indicates that they have not been specified by this stage. However, the absence of expression in the leg discs indicates that the role of *escargot* has diverged between *Drosophila* and *T. biloba*, so is impossible to conclude that the histoblasts are not present at this stage. Identifying the histoblast cells was the motivation for examining *escargot* expression. Because *escargot* is not expressed in these cells, they could not be counted.



**Figure B.4:** *in-situ* hybridized embryos (stage 12) for *escargot*. *escargot* is normally expressed in wing disc, haltare disc, genital disc and 3 leg discs in *Drosophila*, and are expected to be the same in *T. biloba*. However, the expression in the 3 leg discs were not identified by the probe, nor was expression observed in the histoblast cells.